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CONTINUOUS RECOVERY OF SURFACE FILMS OF PROTEIN
FROM DILUTE SOLUTIONS

by



PONG VANANUVAT

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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The undersigned certify that they have read, and
recommend to the Faculty of Graduate Studies for acceptance,
a thesis entitled

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FROM DILUTE SOLUTIONS

submitted by Pong Vananuvat in partial fulfilment of the
requirements for the degree of Master of Science.

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ABSTRACT

A partially immersed rotating glass roller was used to recover surface films of protein from buffered (ph 4.8) solutions of ovalbumin (0.05 to 1.0 per cent), and from whey and blood serum. The roller speeds used were in the range 9 to 192 r.p.m.

Two mechanisms of recovery of protein were observed. The first mechanism was observed with an ovalbumin solution containing traces of unidentified protein fractions and gave denatured protein in the form of an invisible film adhering to the roller surface. The rate of recovery of protein was little affected by the speed of rotation of the roller but was proportional to the concentration of the solution. With the exception of several runs at low speeds, the recovery of protein was less than one milligram of protein per square meter of generated surface area.

The second mechanism was observed with pure ovalbumin solutions at all speeds and at certain combinations of low concentration and high speed with the impure ovalbumin solution. The denatured protein was ejected from the roller on to the free surface of the liquid on the downstream side of the roller, where it accumulated as an insoluble white scum-like film. It appeared that much of the denaturation was occurring on a thin film of liquid being carried over the roller. The ovalbumin recoveries were in the range 0.6 to 7.6 mg./m.², with the greatest recoveries occurring at low speeds and high concentrations.

The speed at which the transition from one mechanism to the other occurred was found to depend on the concentration of the solution, the presence of zinc ions, and to a lesser extent on the history of the solution, and the mechanical characteristics of the apparatus.

A preliminary study of recovery of whey and serum proteins showed that the rotating roller technique caused less surface denaturation of whey than of ovalbumin. Denaturation of serum protein appeared to be a reversible process, as the film of protein tended to redissolve.

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I. INTRODUCTION

Proteins from surface films of monolayers on both water and aqueous solutions (Neurath, 1936). It is generally agreed that this denaturation phenomenon begins with an unfolding of the globular protein molecules, which have spherical, ellipsoidal or polyhedral shapes, to yield a stable protein film or sheet of decreased thickness (Neurath, 1936; Sobotka and Trurnit, 1961). Since proteins are polyelectrolytes this behavior is affected by pH and ionic strength of the underlying subphase. Acids, alkalis, and salts will therefore influence the surface films to varying degrees. The properties and behavior of polypeptide monolayers are also dependent on the composition and distribution of amino acid residues in a molecule. It is therefore expected that proteins of different chemical compositions will behave differently on surfaces, although some of the film properties are quite similar for many proteins (Ikeda, 1962).

Ovalbumin is a relatively labile protein. It is very susceptible to surface denaturation (Bull, 1938; Neurath and Bull, 1936; Wolfe, 1966) and readily forms a surface film. A great many observations have been recorded on the denaturation and formation of uni- and multi-molecular layers of ovalbumin films at liquid-air interfaces (Bull and Neurath, 1937; Neurath and Bull, 1937; Gorter, 1937; Bull, 1938; Ikeda, 1962). Abundant data are available on the transfer of built-up protein and fatty acid films to microscope slides (Langmuir et al., 1937; Langmuir, 1938; Ormond, 1939; Langmuir, 1939; Langmuir and Schaefer, 1939). No reports of transfer of protein films from liquid to solid surfaces

more recent than 1939 have been reported. There are very few data available on the kinetics of surface denaturation of proteins, and apart from the study of Bull (1937) it appears that no work has been done on the technological aspects of recovery of protein films from the surface of protein solutions.

Bull's experiment of 1937 gave data on the kinetics of surface denaturation of egg albumin. A porcelain drum rotating at known speeds was used to dip into a solution of ovalbumin. The amount of ovalbumin denatured per unit area of the roller surface was determined as a function of the rate of creation of new surface as well as of the concentration of the solution. It was found that both greatly affected the amount of denaturation.

The study of Bull (1937) was limited to a narrow range of speed and was concerned primarily with the chemical point of view. A thorough study of this process should be of importance because it should throw light upon the technological aspects of recovery of proteins from dilute solutions on an industrial scale.

II. REVIEW OF LITERATURE

1. The Properties of Protein Monolayers

Although proteins themselves are frequently very soluble in water, coherent monolayers which they form on surfaces are insoluble. This shows that a change of structure has occurred during the spreading of the protein. Molecules which are wholly hydrophilic have no tendency to spread on the surface of water. The very high solubility of certain proteins thus indicates the absence of hydrophobic groups on the outside surface of their molecules, yet their spreading tendencies suggest that a large number of hydrophobic groups are exposed when the molecule is unfolded (Langmuir, 1938).

Gorter and Philippi (1934) found that the thickness of an ovalbumin monolayer, as calculated from the area covered by a weighed amount of the protein, was of the order of $10\text{\AA}$. This calculation can only be approximate, since it assumes that all the protein used has spread to produce the monolayer. However, this finding was supported by the x-ray studies of Astbury et al., (1938).

Protein monolayers are easily and reversibly compressible (Langmuir, 1938). The properties of the protein monolayers seem to show that the monolayer is a kind of duplex film which consists of polypeptide chains of amino acids attached by hydrophobic groups to the air-water interface at intervals along their lengths. The hydrophilic parts of the polypeptide chains remain in contact with and are surrounded by water, but are not free to go far from the surface because they form parts of chains which are still attached to the surface. When the film

is compressed there may not be room enough for all the hydrophobic groups on the surface so that some are driven down into the water just below the surface. Since these hydrophobic groups tend to coalesce or adhere to each other, they are at least partly responsible for the viscosity and rigidity of the monolayers.

Bull (1947) described two methods of measurement of surface viscosity. The passage of the film through a small channel can be observed or the oscillations of a disk in the surface can be measured. Work done by Langmuir and Schaefer (1939) showed that the film viscosity increased greatly as the film pressure was increased and further that the various proteins at the same film pressure showed tremendously different viscosities. The high viscosities were explained as interaction of the polar groups of neighboring spread protein molecules.

2. The Spreading Process of Protein Monolayers

Ormond (1939) divided proteins which can be spread into two different classes:

Class A consists of those which spread throughout the whole pH range including their pH-stability range. Examples of A-proteins are: egg albumin, serum albumin, lactoglobulin, insulin, and pepsin.

Class B consists of those which spread only on a very acid (pH about 1) or a very alkaline substrate (pH about 13). An example of this class is the hemocyanin of Pulmonaria vulgaris.

The spreading process may be considered as being divided into the following steps (Figure 1):

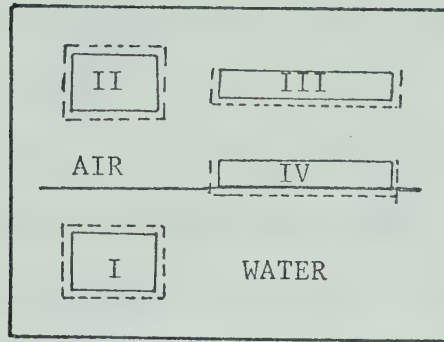


Figure 1.--Spreading process of protein.
From Ormond (1939).

I - II. The protein molecule with its thin water film is brought from the interior of the solution into the air.

II - III. The shape of the molecule is change to the shape it will have in the spread condition. Then its lower surface is hydrated. It may also be hydrated at other points such as those situated along its circumference.

III - IV. The hydrated plate-like molecule is laid on the water surface, and in this condition it is insoluble.

Gorter (1937) summarized the factors which counteract the spreading tendency. One of the most important factors is the number of ionizable groups on the surface of the protein. Everything which prevents the ionization of these groups increases the spreading tendency. This can be shown as follows:

a) On increasing the ionic strength of solutions of proteins such as ovalbumin, which have a large number of both kinds of ionizable groups, spreading occurs almost immediately. As well, film formation occurs at pH values where there is no spreading at all in the absence of electrolytes. According to Neurath and Bull (1938), anions are more

effective in reducing the spreading time of positively charged protein than are cations, and polyvalent anions are more effective than univalent anions. Cations have a greater influence on the alkaline side of the isoelectric point than do anions.

b) The studies on artificially prepared complex proteins (by the addition of bivalent acids are amines which are attracted to the $-\text{NH}_3^+$ or $-\text{COO}^-$ groups) e.g., ovalbumin-tartrazin, indicate that the properties of the natural proteins also differ according to their composition. The simple proteins, such as clupein, do not spread, because the "upper" surface of the film of this protein contains too many $-\text{NH}_3^+$ polar groups and is therefore too soluble. Other proteins, e.g., myosin, fibrinogen, and also heat-denatured ovalbumin, do not spread because the lower surface contains too few free polar groups, for it these proteins are split into somewhat smaller particles by proteolytic enzymes, more $-\text{COO}^-$ and $-\text{NH}_3^+$ groups are made free and the product has normal spreading tendency.

3. Transfer of Surface Films onto Solid Surfaces

The technique of film transfer was developed by Blodgett in 1934. This is probably the most important single development in the study of surface films of protein since the introduction of the spreading trough by Langmuir in 1917. The protein was introduced onto the surface of water by means of a platinum wire. This protein film was transferred to a plate about the same size as a microscope slide which was made of highly polished chromium plated brass, made hydrophobic when required by treatment with barium stearate.

a. Monofilms

Langmuir (1937) used Blodgett's method to transfer a single

monolayer of protein onto a plate. He found two methods of transferring monolayers. In method A, the plate was held in a vertical plane and lowered into water through the protein film. Any residual protein was removed from the liquid surface. When the plate was raised out of the water, the protein film was then applied to the surface of the liquid. Upon raising the plate out of the solution the protein film was not yet in contact with the plate but lay on a water film. As the water dried, the protein film became attached to the plate.

b. Multiple films

There are many types of multiple films, but only one type will be mentioned here as it is the only type of interest in the present study.

For classification of the types of film obtained the following letters were used: P for plate, R for the hydrocarbon surface of barium stearate layers, A and B for protein layers produced by Methods A and B above respectively. Thus PRAB was a film prepared by placing the B film on an A film which had been deposited on a hydrophobic plate PR, as shown in Figure 2.

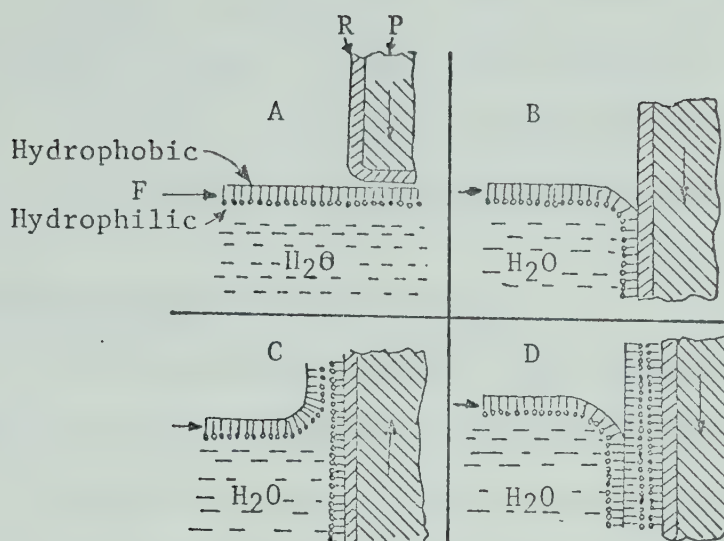


Figure 2.--Formation of multilayers of molecules.
From Langmuir et al. (1937).

The plate (Figure 2A) was lowered into water covered by a protein film (Figure 2B). The plate was then withdrawn (Figure 2C) and dried, leaving two layers of protein on the surface of the plate. If a PRAB film was lowered into pure water or into water covered by a protein film, even under 30 dynes per cm. pressure, the B film was ejected from the plate on to the water surface.

The addition of 10 grams per litre of zinc chloride to the water prevented the separation of the B from the A layer and made it possible to continue adding AB layers indefinitely giving PRABABAB..... films (Figure 2D). In this way 30 layers were built up without difficulty.

It was concluded by Langmuir et al. (1937) that there were some essential differences between A layers and B layers. One of the differences was that B layers which lay upon A layers were ejected onto the water surface when the plate was immersed in water in the absence of

zinc ions, whereas A layers on B layers were not ejected. The methods used to form the layers indicated that A layers were inverted in their formation, whereas B layers were not inverted. Thus the outer surfaces of the A and B layers should be hydrophilic and hydrophobic respectively.

4. Surface Denaturation of Proteins

Bull and Neurath (1937) stated that the process of surface denaturation seems to consist of at least two reactions:

a) The structure of the protein molecule is changed in some manner which results in a far more hydrophobic molecule than the natural protein. Neurath (1936) has described this as being an irreversible unfolding of the protein molecule at the surface.

b) The second reaction consists in the precipitation or coagulation of the denatured protein.

Bull and Neurath (1937) also postulated that the rate of surface denaturation by shaking would be governed by four reaction velocities:

1. The rate of diffusion of protein molecules from the bulk of the solution to the surface.

2. The rate of spreading and denaturation of the protein on the surface.

3. The rate of formation of a new surface.

4. The rate of precipitation of denatured protein in the bulk of the liquid.

Bull (1937) developed an apparatus for the study of protein films on protein solutions which yielded somewhat more quantitative results. A roller of known dimensions was dipped into an ovalbumin solution and rotated at known speeds. With this technique it is possible to measure the rate and extent of creation and destruction of air-protein solution interface. The amount of protein surface-coagulated per unit surface area as a function of speed of rotation was calculated from the amount of protein surface-coagulated in a given time. The thickness of the film of denatured protein at the surface was calculated from the specific volume of the protein. It was found that the thickness of the film calculated in this manner was a smooth function of the speed of rotation of the drum.(Figure 3).

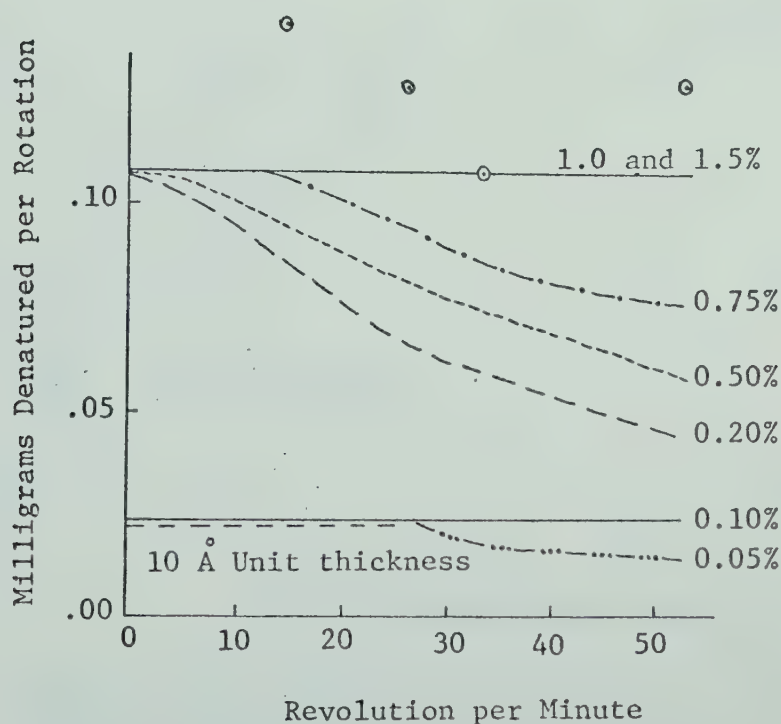


Figure 3.--Amount of denaturation per rotation (surface area per rotation 171.6 sq.cm.) as a function of speed for several concentrations used. From Bull (1937).

It was estimated that the thickness of the film on a quiescent solution (by extrapolation of the thickness of the film to zero speed of rotation) for 0.1 per cent solution was $10.6 \text{ \AA}$, and this thickness was independent of the speeds of rotation. Below 0.1 per cent solution, the calculated thickness was dependent on the speed of rotation. With concentrations greater than 0.1 per cent, the thickness of the film increased rapidly with an inflection at 0.2 per cent (Figure 4).

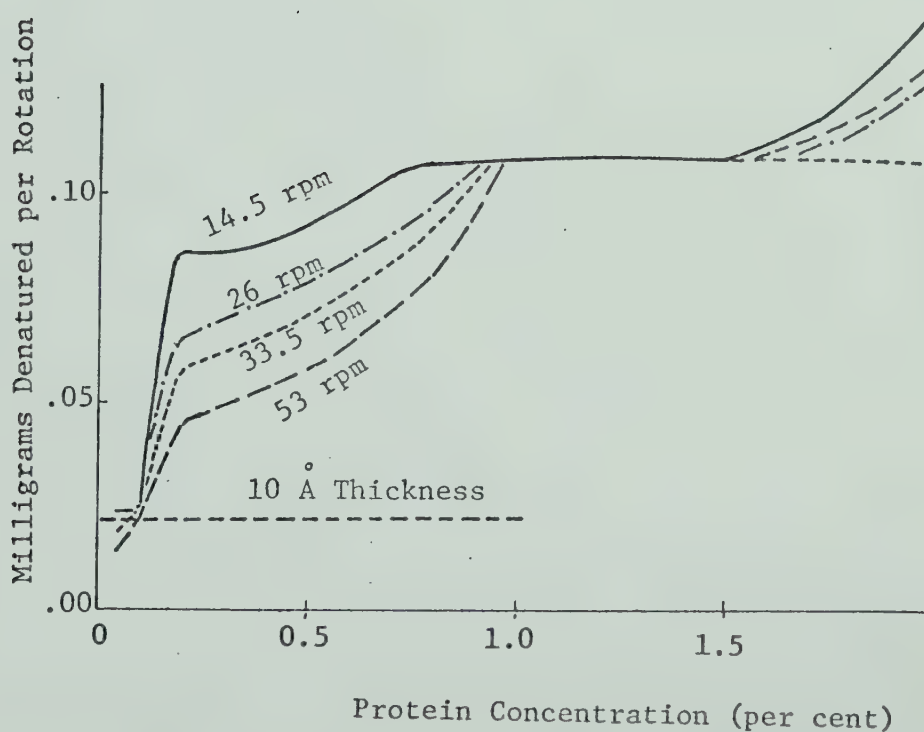


Figure 4.--Amount of denaturation per rotation as a function of protein concentration for several speeds of rotation. From Bull (1937).

It was assumed that additional protein was adsorbed under the first $10 \text{ \AA}$ layer. This second layer of protein contained as a limit 3.5 times the amount of protein in the first layer and was probably in an undenatured state.

However, Bull (1937) reported neither the nature of the buffer system used and its ionic strength, nor the depth of the protein solution in the container. Also the effect of the period of rotation at different roller speeds was not taken into consideration.

III. EXPERIMENTAL

1. General Approach

The purpose of this investigation was to study the recovery of protein from dilute solutions using a rotating roller. The general method employed was to determine the amount of protein on the surface of the roller after it had been rotated in a protein solution. The effects of roller speed, pH, concentration, period of rotation, zinc ions, ionic strength, and depth of protein solution were studied.

2. Protein Preparation

a) Preparation of 'ovalbumin

Three batches of ovalbumin (ovalbumin 1,2 and 3) were prepared from fresh hen's egg by the method of Kekwick and Cannan (1936) and recrystallized four times. It was dialysed in cellophane tubing against distilled water in the cold to the absence of sulfate. Protein which precipitated upon dialysis was removed by filtration through Whatman No. 1 filter paper. The filtrate was lyophilized, and the product stored at -18°F in wide-mouth plastic bottles.

Moisture determination of the three preparations were made according to the method of the Association of Official Agricultural Chemists (A.O.A.C., 1965). The homogeneity of the three batches of ovalbumin were checked by polyacrylamide gel electrophoresis (Davis, 1964).

b) Preparation of globulin fraction

Egg white globulin was obtained by adding an equal amount of a saturated solution of NaSO_4 to 1:1 dilution of egg white with distilled

water (Kekwick and Cannan, 1936). The precipitated globulin fraction was removed by filtration onto Whatman No. 1 filter paper. The precipitate was then dissolved in phthalate buffer at pH 4.8 (ionic strength of 0.067) and dialysed in cellophane tubing against the same buffer in the cold until free of sulfate ion. This protein fraction was lyophilized and stored at -18°F in wide-mouth plastic bottles.

c) Preparation of whey

Fresh raw skim milk was heated to $30 \pm 2^{\circ}\text{C}$. Slow addition of 0.1N HCl with constant stirring was made over the period of half an hour to bring the final pH to 4.60. It was centrifuged at about 1000 g for 15 min. to remove precipitated casein. The supernatant fluid contained the whey proteins.

d) Preparation of serum protein

Animal blood, fresh from a packing plant, was allowed to clot overnight at room temperature. The clot was then removed by centrifugation at about 1000 g for 15 minutes. The supernatant serum was used immediately.

3. The Rotating Roller Devices

Two sets of the rotating roller device, a one-tray device (device 1) and multi-tray device (device 2) were made. Device 1 was used in the experiments reported in Part I of the results, and device 2 in those reported in Part II.

a. The one-tray rotating roller device (device 1)

A rotating glass roller as shown in Figure 5 and 6 was employed to remove a monolayer or multilayers of protein from surface of the protein solution. The rotating roller had the following dimensions:

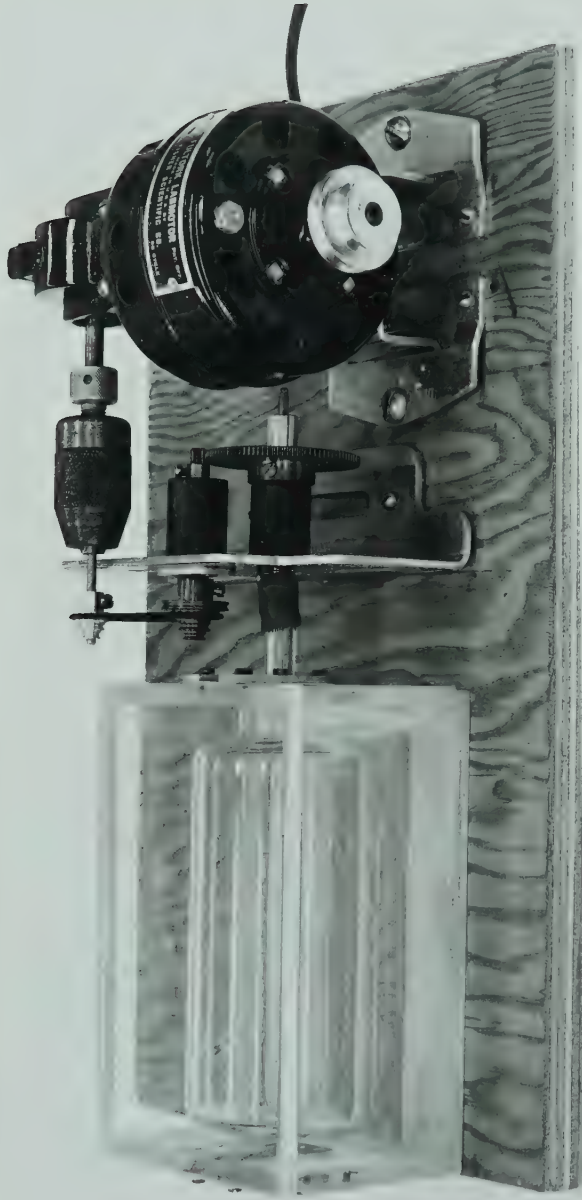


Figure 5.--One-tray rotating roller device.

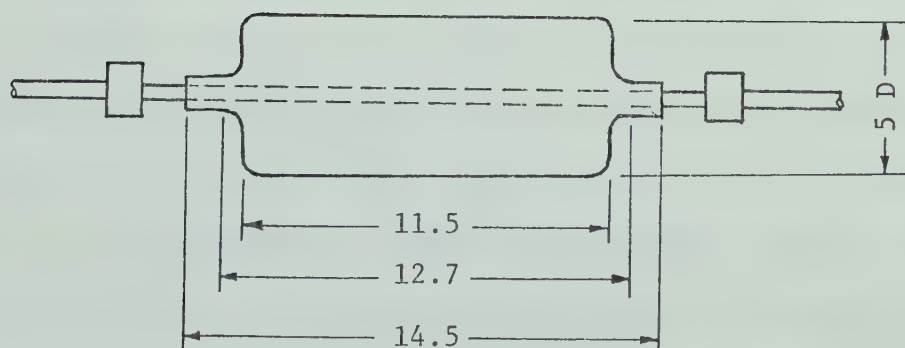
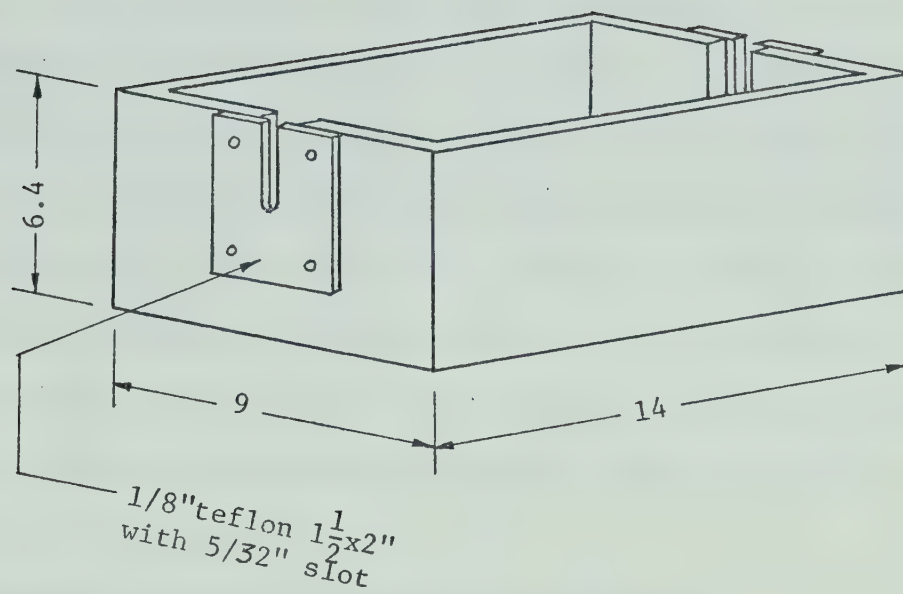


Figure 6.--Diagram of glass roller and lucite tray, all dimensions in centimeters, except where noted.

diameter 5.0 cm., circumference 15.7 cm., length 11.5 cm., giving a total area of wet surface of 181 sq. cm. for each revolution. The roller was placed in position in the lucite container which contained a given quantity of protein solution. The container, made of 1/4 in. lucite, had the following dimension: length 14.0 cm., height 6.4 cm., width 9.0 cm., the distance between the lower surface of the roller and the bottom of the container 1.0 cm. Samples of 200 ml. of protein solution were used giving a liquid depth of 2.0 cm. The motor used to rotate the roller gave a range of speed through a combination of gears and pulleys. The selected speeds of rotation were 9, 13, 18, 26, 33, 42, 53, 69 and 94 r.p.m.

b. The multi-tray rotating roller device (device 2)

This new design as shown in Figure 7 was more versatile than device 1 as four rollers could be simultaneously rotated in series through connections between the shafts of the rollers. The dimensions of the containers were the same as described in section 6a (page 16), but the rotating rollers had the following dimensions: diameter 5.0 cm., length 11.0 cm. The total area of wet surface was 173 sq. cm. per rotation. A small synchronous geared motor (output speed 60 r.p.m.) was used with a system of interchangeable gears to drive the rollers (Table 1). The combinations of the gears were arranged to allow 5% increments between the lowest and the highest speeds of the final drive which were 17.9 and 201.5 r.p.m. respectively. A slight modification of gear box was later made to reduce the lowest speed to 9 r.p.m. Samples of 200 ml. of solution were used giving the liquid depth of 1.9 cm., except when the volume was an experimental variable. The selected

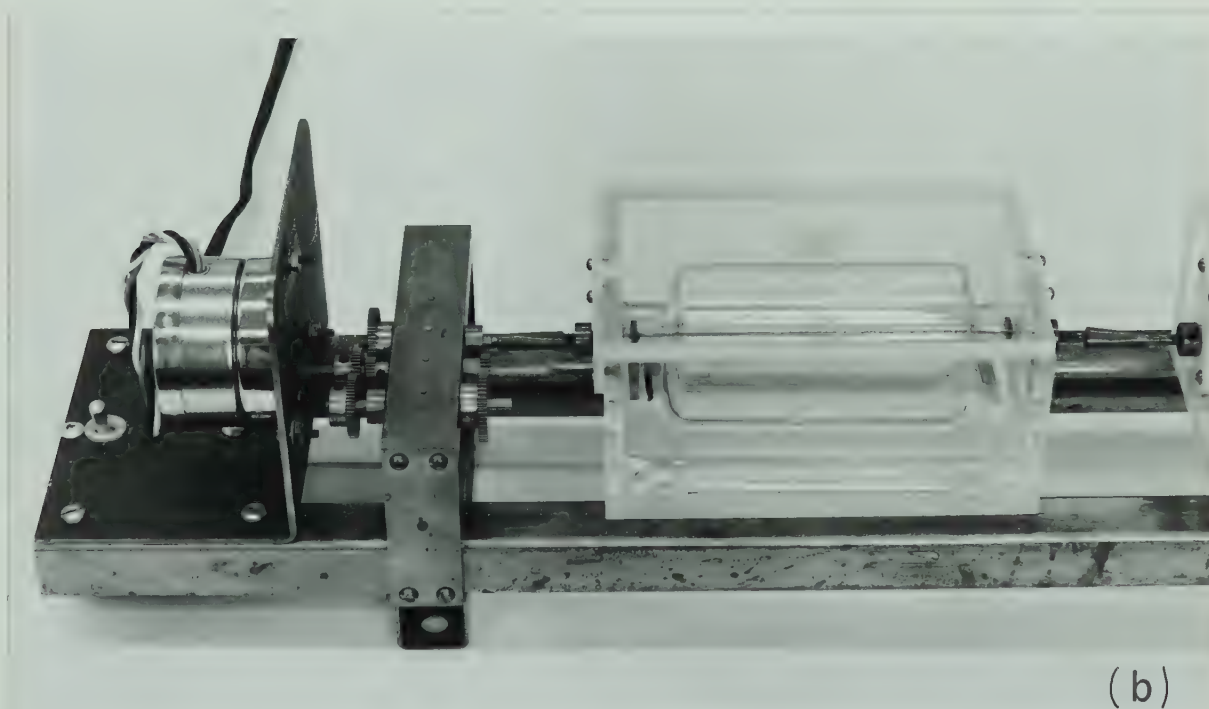
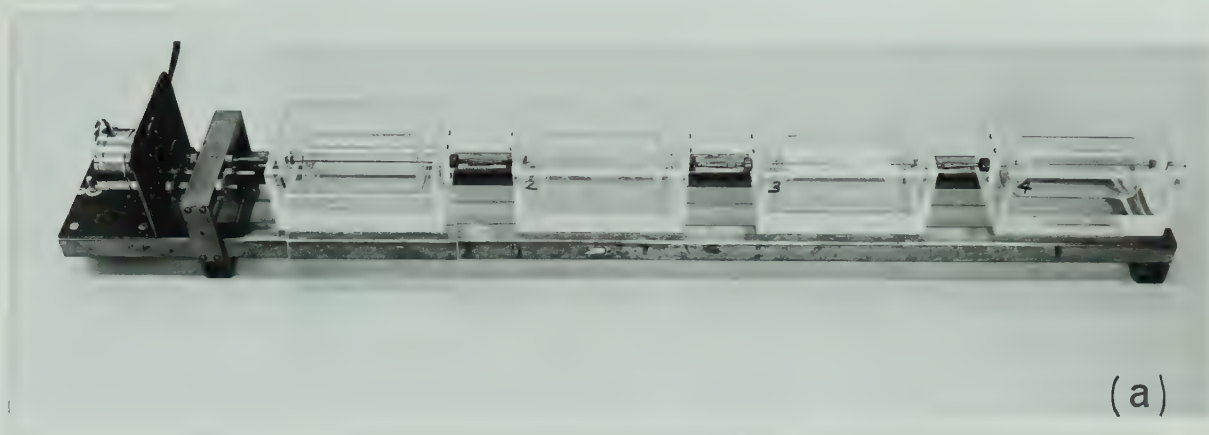


Figure 7.--Multi-tray rotating roller device.
(a) General view of the apparatus.
(b) Detail of geared drive.

Table 1.--Relationship between gear ratios and final speeds of the rotating roller (device 2).

Primary gear ratios ^a		Roller shaft speed (r.p.m.)		
		Find gear ratio ^a		
Set one	Set two	28/64	Direct	64/28
44/46	40/56	17.9	41.0	93.7
40/56		18.8	42.9	93.7
46/44	40/56	19.6	44.8	102.4
44/56		20.6	47.1	107.7
46/56		21.6	49.3	112.6
40/46		22.8	52.2	119.1
40/44		23.8	54.4	124.4
44/46		25.1	57.4	131.3
40/40		26.3	60.0	137.1
46/44		27.5	62.7	143.4
44/40		28.9	66.0	150.9
46/40		30.2	69.0	157.6
56/46		32.0	73.1	167.0
56/44		33.4	76.4	174.6
44/46	56/40	35.1	80.3	183.5
56/40		36.8	84.0	191.9
46/44	56/40	38.5	87.8	201.5

a) Gear ratios are expressed as (number of teeth on driving gear) / (number of teeth on driven gear). The arrangements of the various combinations of gears are shown in Figure 23 in Appendix I (page).

speeds of rotation used in experiments with device 2 varied, and are specified in each set of experimental results.

4. Procedure Used in the Rotating Roller Experiment

The following procedure was used throughout this investigation unless otherwise specified. All experiments were carried out at ambient temperature.

Lyophilized ovalbumin was added to phthalate buffer at pH 4.8 (the iso-electric point of ovalbumin), and ionic strength of 0.067. The solution was then filtered through Whatman No. 1 paper to remove undissolved protein, and a sample taken to determine the concentration of the filtered protein solution. The required volume of the solution was transferred to the container, and the dry glass roller was set in place. The whole apparatus was covered with a lid to minimize water loss through evaporation. The roller was rotated for the required time at the lowest speed for the first run. After each run, the roller was removed and a new one was set in place in the same solution, and rotated at the next higher speed. The protein recovered on the roller surface was dissolved in 25 ml. of 0.1N NaOH. This was done in a shallow tray designed such that the surface of the roller just touched the surface of NaOH solution (Figure 8). The roller was rotated by hand several turns until the protein deposited on the surface of the roller dissolved completely in NaOH. The protein concentration of the resulting solution was determined according to the method of Lowry et al. (1951). The absorbance at 750 mμ was determined in a Beckman Model DBG spectrophotometer using a 1-cm. cell. All determinations



Figure 8.--Shallow tray for removing protein from roller surface.

were carried out in duplicate. The standard curves for concentration measurement of ovalbumin 1, 2 and 3 were constructed by dissolving a weighed amount of lyophilized proteins of known moisture content in 0.1N NaOH to attain the desired concentrations.

During the work it was found that the amount of liquid which remained on the roller when the roller was lifted from the solution contained significant amounts of undenatured protein. The technique of determination of the amount of denatured protein was therefore modified as follows: the dry roller was weighed before the run, and after the run it was reweighed to determine the amount of solution remaining on the roller. The amount of protein in the adhering solution was calculated from the weight of the solution and the concentration, and this amount of protein was subtracted from the total protein as determined spectrophotometrically.

5. Determination of Thickness of Liquid Layer on the Surface of the Roller

The glass roller was cleaned and left to air-dry for about one hour. The dry roller was weighed with a support on an analytical balance. The roller, after being rotated in the protein solution, was removed in such a way that no scum deposited on the surface of the roller. The bottom half of the roller and both ends were blotted dry. The roller was then weighed again with the same support. The process of removing the roller from the solution until to the step of weighing was done as fast as possible in order to avoid evaporation of water from the surface of the roller. The roller was left to air-dry again for about one hour and then reweighed. The difference between the second and the third weighing was the amount of water in the liquid layer retained on the top half of the roller surface.

IV. RESULTS AND DISCUSSION

The results and discussion of this investigation are separated into two parts. Part I covers ovalbumin 1 in device 1, and Part II covers ovalbumin 2 and 3 in device 2.

PART I

1. Protein Recovery as a Function of Roller Speed in Unbuffered Solutions

Some experiments were done on ovalbumin 1 dissolved in distilled water at pH 6.75-6.80. The rate of protein recovery (mg./min.) is plotted in Figure 9 against speed of rotation for three concentrations of solution. Apparently the speed of rotation has only a slight effect on the rate of protein recovery at concentrations of 0.07, 0.34, and 0.71 per cent. The average rate of recovery at each of the three concentrations is plotted against concentration in Figure 10 (lower curve) which shows that the rate of protein recovery is approximately proportional to the concentration of the solution. An explanation of this phenomenon could be that the roller removes all the protein film from surface of the solution and hence the amount recovered would be proportional to the rate of diffusion of the protein from the bulk of the solution to the surface. This observation is in good agreement with the results of Bull and Neurath (1937) who studied the denaturation of ovalbumin by shaking. They found that the overall rate of coagulation of this protein depended on the rate of diffusion of soluble protein into the surface monolayer.

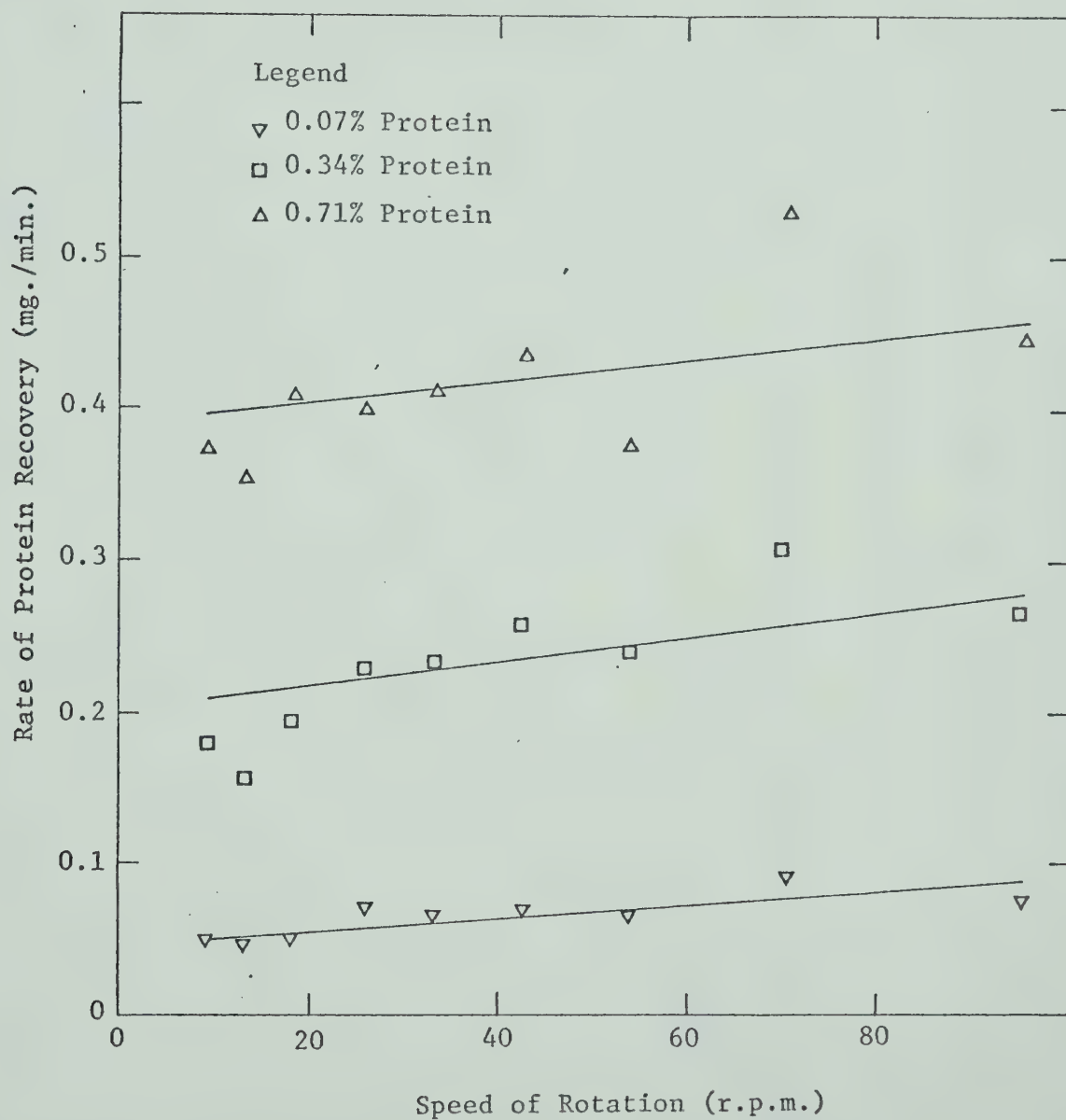


Figure 9. Effect of speed of rotation on rate of protein recovery of ovalbumin 1 in unbuffered solutions (pH 6.75) for several concentrations of protein.

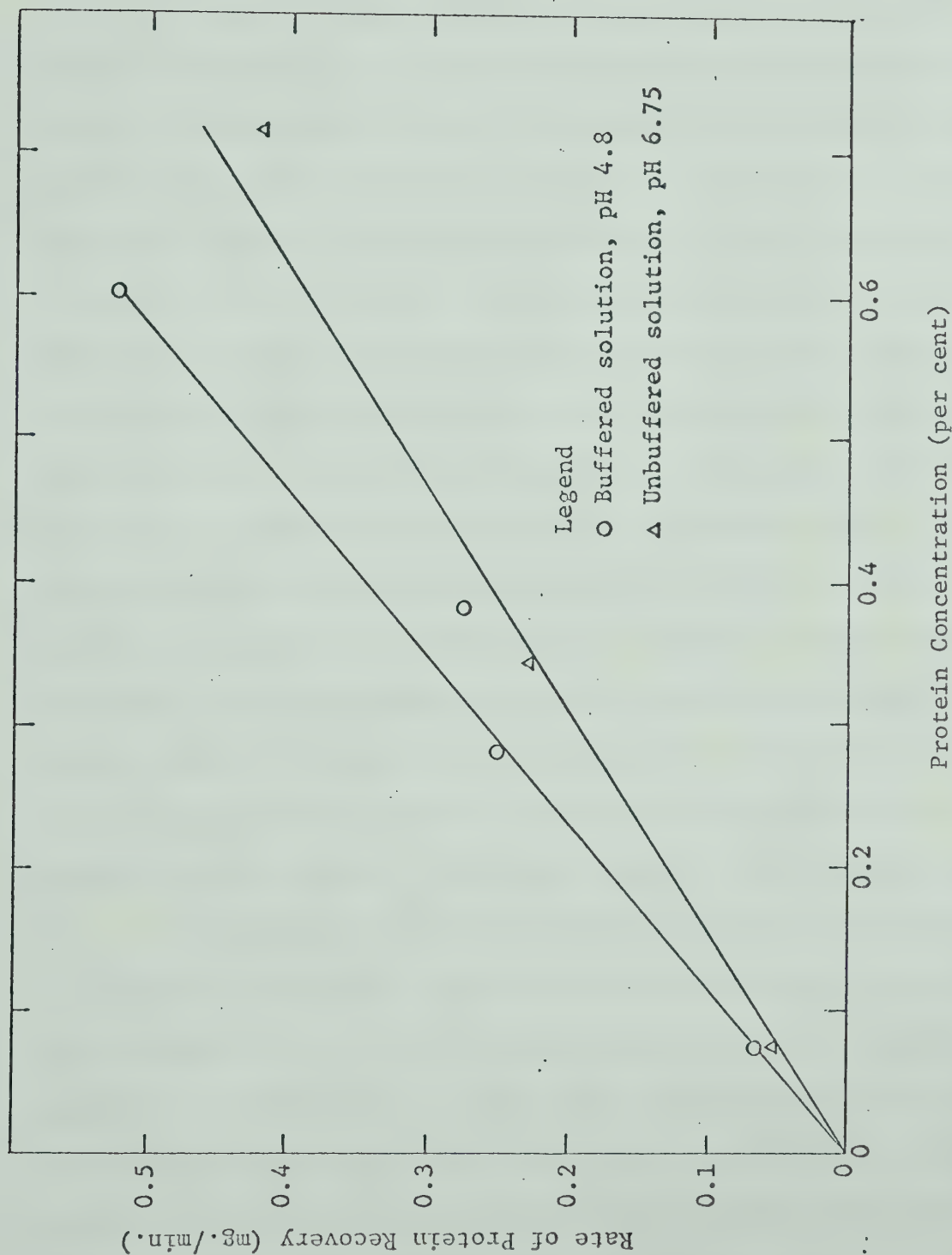


Figure 10.--Effect of protein concentration on rate of protein recovery of ovalbumin 1 in unbuffered and buffered solutions. Each point represents the average of rates at all nine speeds. The rate constants are 0.86 and 0.64 (mg.)/(min.)(per cent protein) for the buffered and unbuffered solutions respectively. No corrections have been made for the effect of protein in the solution remaining on the roller when the roller was removed from the tray.

2. Protein Recovery as a Function of Roller Speed in Buffered Solutions

Figure 11 shows the rate of protein recovery (mg./min.) vs. speed of rotation (r.p.m.) for four concentrations of ovalbumin 1 dissolved in the buffer at pH 4.8. The speeds of rotation had very little effect on the rate of protein recovery at all concentrations except the run at 0.06 per cent concentration and 94 r.p.m. . The average rates of protein recovery from the buffered solution were somewhat higher than those for the protein dissolved in water (see Figure 10). The results obtained here agree well with the works of Wu and Ling (1927), and Bull and Neurath (1937) who observed that the rate of surface denaturation by shaking of ovalbumin was maximum at its isoelectric point. Lindau and Rhodius (1935) determined the amount of ovalbumin adsorbed on surface of quartz powder and found that at values of pH other than the isoelectric point, the ovalbumin molecule swelled and occupied a much larger volume. The maximum surface density is therefore at the isoelectric point, and the removal of the surface layer should lead to a maximum rate of recovery. This is as observed in the present study.

A relatively high rate of protein recovery (1.63 mg./min.) was observed for 0.06 per cent solution run at 94 r.p.m., whereas at all lower speeds, the rate of protein recovery was much less (average 0.07 mg./min.). A stable film of protein was observed on the surface of the solution at the down side of the roller soon after this run had started. This film deposited onto the roller surface when it was lifted from the solution. For the purposes of this thesis, the stable film of protein is henceforth described as "scum" or "scum-like film". A mechanism for its formation is discussed in section 4 (page 28).

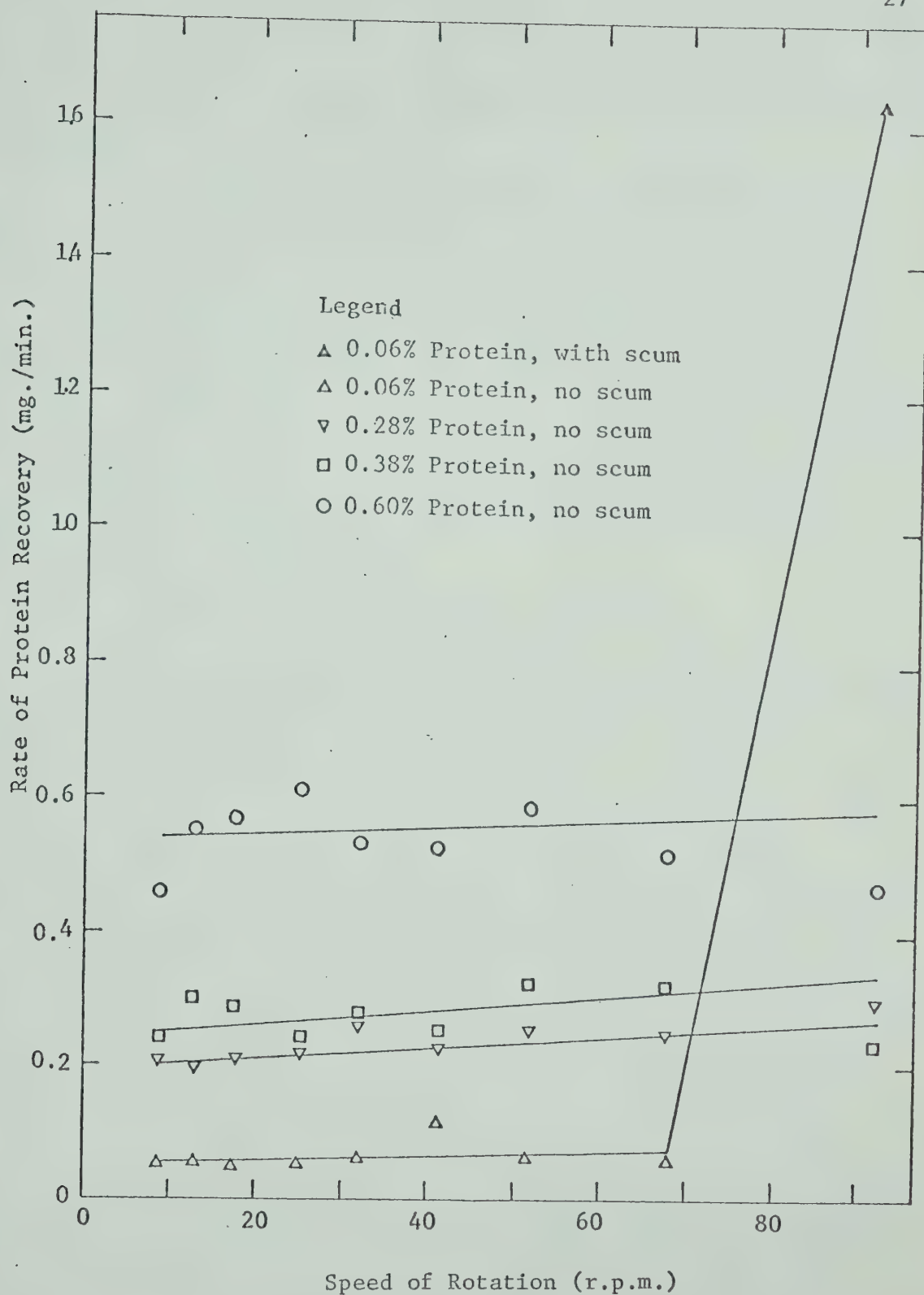


Figure 11.--Effect of speed of rotation on rate of protein recovery of ovalbumin 1 in buffered solutions (pH 4.8) for several concentrations of protein. No corrections have been made for the effect of protein in the solution which remained on the roller surface when the roller was removed from the tray. (Later experiments indicate that approximately 80 and 20 per cent of the total protein recovered has been surface denatured in the runs with and without scum formation respectively).

3. Recovery of Protein per Unit Area and Its Thickness as a Function of Speed

The amount of protein recovered per square meter of the roller surface (R) was obtained from the equation, $R = M/A$, where M is the mass of the recovered protein, and A is the total surface area generated by the rotating roller. The volume of the recovered protein (V) can be calculated from the equation, $V = M/D$, where D is the density of the denatured protein. The density was taken to be 1.30 gm./cm.³ (Neurath and Bull, 1937). The thickness of the protein (T) film is then given by the equation, $T = V/A$.

Protein recovery per unit area (mg./m.²) and thickness (Å) are plotted against speed of rotation in Figures 12 and 13 respectively. Both the protein recovery and the average thickness decreased as speed was increased (except for 0.06 per cent solution at 94 r.p.m.). This is because the rates of protein recovery were virtually independent of speed, but the rate of generation of surface area is directly proportional to speed. According to Gorter and Philippi (1934), and Neurath (1936), the thickness of a monolayer of a film of ovalbumin is equal to 10.6 Å. Therefore at 9, 13 and 18 r.p.m., more than one monolayer was removed from the 0.60 per cent solution (Figure 13). For the 0.06 solution at 94 r.p.m. the average thickness of the protein film removed was 86 per cent of that of a monolayer. This indicates that almost one monolayer was formed, whereas at 69 r.p.m. at this concentration, only 3 per cent of the surface was covered with protein film.

4. The Proposed Mechanisms of Formation of Scum-like Film

The experiments in section 2 showed that two mechanisms of protein recovery occurred. At low speeds the protein film absorbed firmly onto the surface of the roller, possibly in the form of RABAB.... layers,

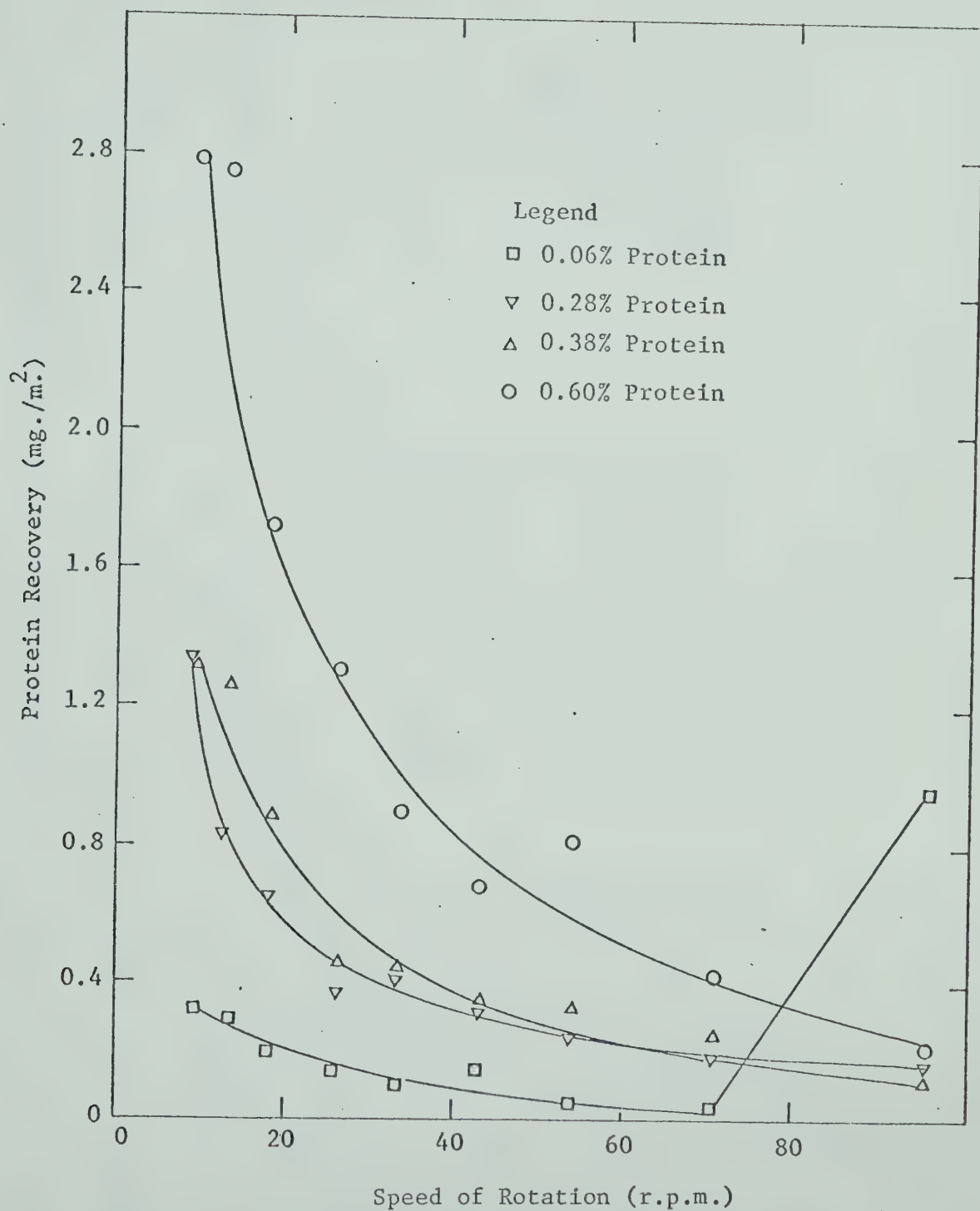


Figure 12.--Effect of speed of rotation on protein recovery of ovalbumin 1 in buffered solutions (pH 4.8) for several concentrations of protein. No corrections have been made for the effect of protein in the solution remaining on the roller when the roller was removed from the tray.

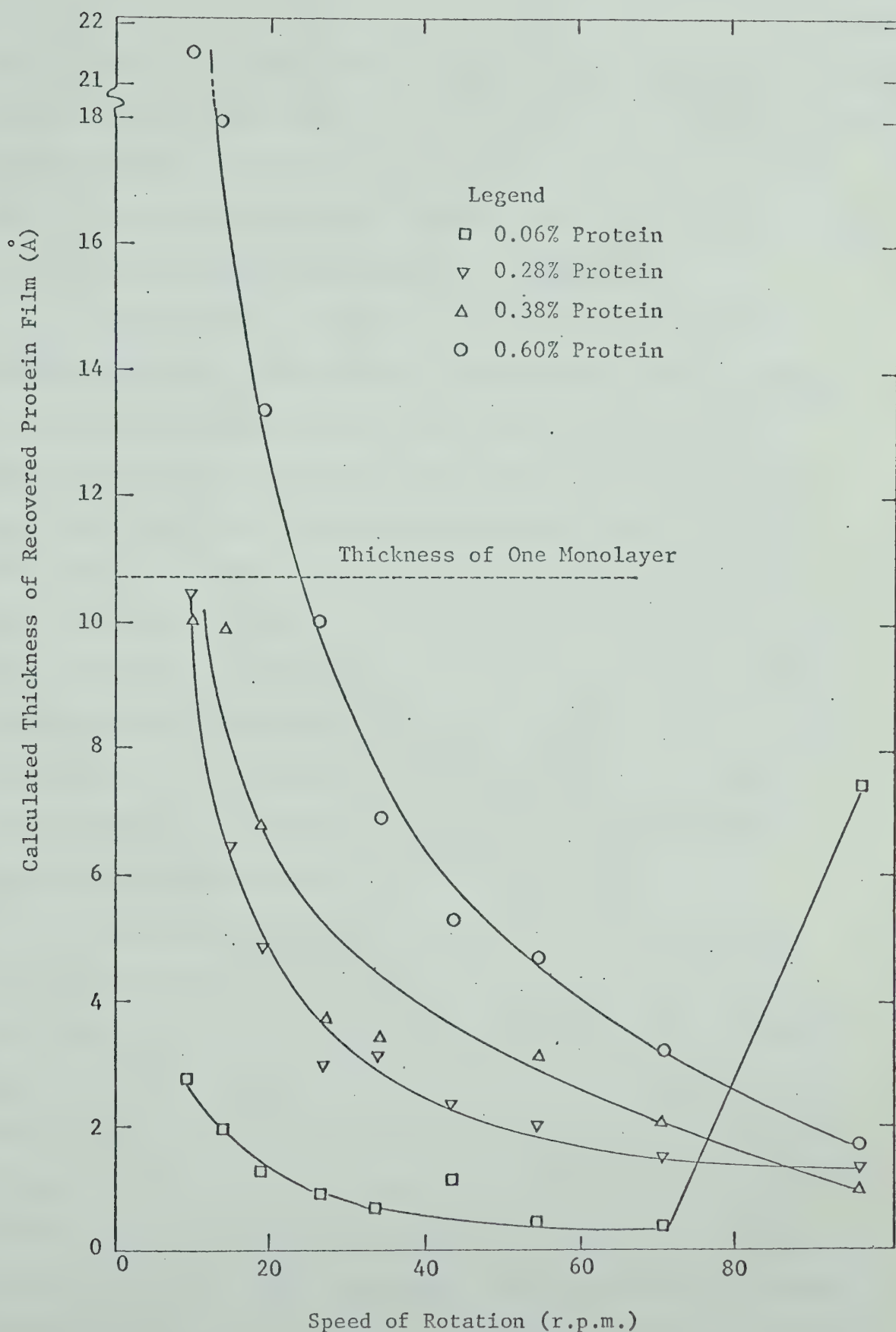
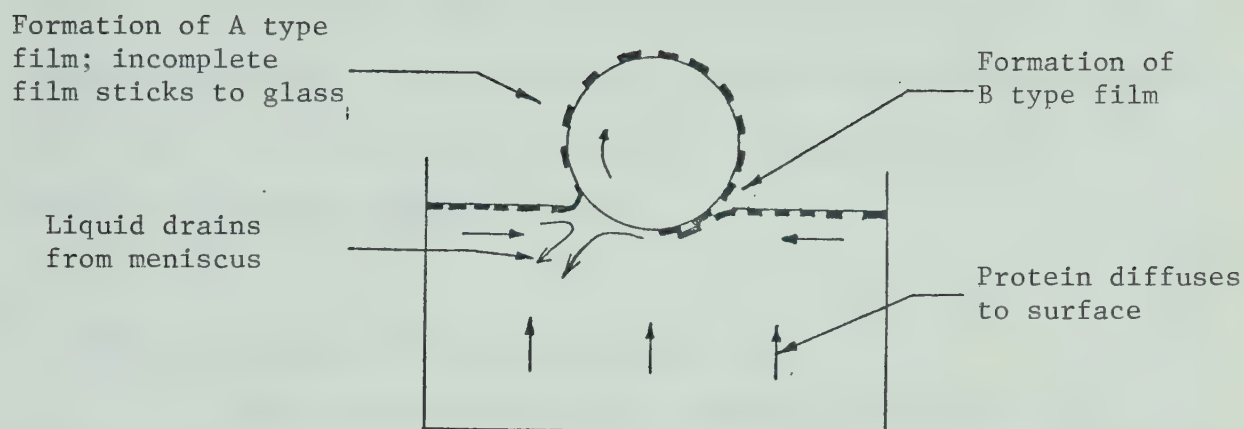


Figure 13.--Effect of speed of rotation on calculated thickness of recovered protein film for ovalbumin 1 in buffered solutions (pH 4.8) for several concentrations of protein. No corrections have been made for the effect of protein in the solution remaining on the roller when the roller was removed from the tray.

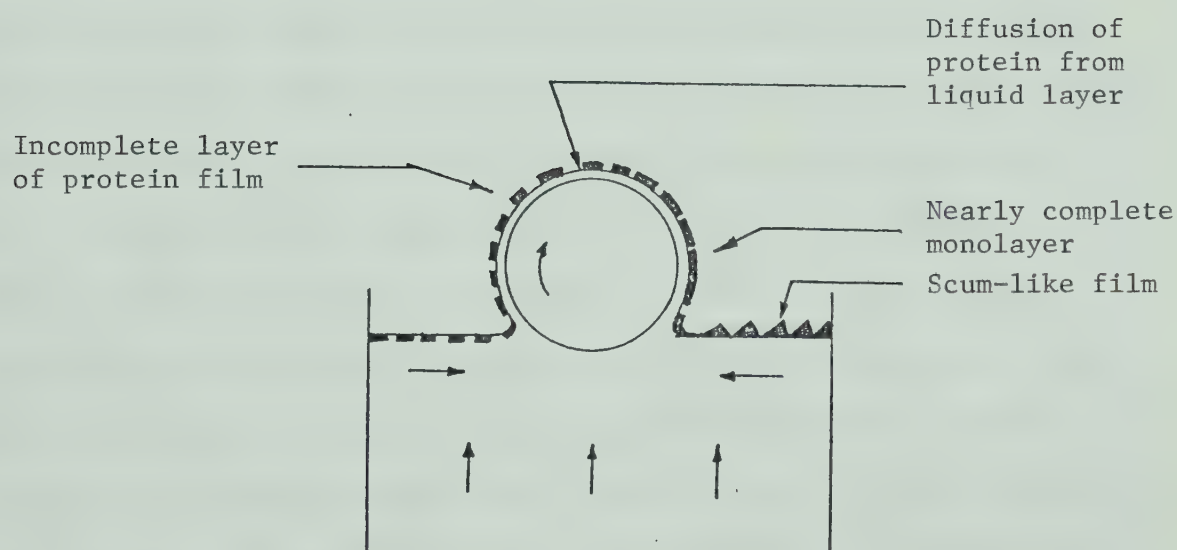
where R refers to the glass roller surface, A and B refer to the two types of protein film as described in section 4b (page 7). At high speeds the recovered protein was observed on the surface of the solution in the form of scum-like film instead of adsorbing onto the roller surface. The two mechanisms are shown diagrammatically in Figure 14.

For the film to adhere to the roller surface, solution must drain completely from meniscus. As the roller speed increases, the amount of solution moving into the meniscus (due to movement of liquid surface and roller surface) increases. The increased flow of liquid would then cause a separation between the protein film and the roller surface, so that a film of liquid would be carried over the roller, as shown in Figure 14b. The speed of rotation at which this separation takes place would depend on the properties of the surface film (especially surface strength) and the strength with which it adheres to the roller. It is expected that the properties of the liquid (particularly viscosity and density) will also affect the speed at which separation takes place.

As the film is carried over the roller surface, further diffusion of protein from the liquid film to its surface would take place, and so increase the overall amount of denaturation. Because the film is no longer in contact with the roller, it would not be carried down below the surface of the solution in the tray, but it would float out onto the surface of the solution on the down side of the roller. Because the ends of the tray prevent the film from moving away from the roller, the film becomes compressed. This accumulation of protein film on the surface of the solution would render it visible as a scum-like layer. Calculations based on the amount of ovalbumin required for



(a) Low speeds; no scum formation.



(b) High speeds; scum formation.

Figure 14.--Proposed mechanisms of protein recovery.

a formation of one monolayer (approximately 1 mg./m.^2 , Langmuir and Schaefer, 1939) showed that $2 \times 10^{-3} \text{ cm.}^3$ of a 0.06 per cent solution per cm.^2 of the roller surface contained enough protein to form one complete monolayer. Therefore a film of liquid with a thickness greater than $2 \times 10^{-3} \text{ cm.}$ (equal to $2 \times 10^{-3} \text{ cm.}^3 / \text{cm.}^2$) would contain sufficient protein to give the observed results.

5. Effect of Zinc Chloride on Film Formation

It has been shown that the spreading of ovalbumin on surface of the solution is affected to a great extent by divalent cations in the solution (Gorter, 1937; Neurath and Bull, 1938). These cations have been shown by Langmuir (1939) to assist the built-up of multi-layers of protein by preventing the ejection of the fresh monolayer onto the surface of the liquid on the down stroke of a glass slide used to deposit protein films. Hence, the effect of addition of ZnCl_2 to protein solutions was examined. Figure 15 shows protein recoveries for solutions of 0.06 per cent protein (without zinc ions) and for 0.05 and 0.03 percent protein (0.05 percent zinc chloride). No scum was observed at speeds lower than 94 r.p.m. for the 0.06 per cent solution. The addition of the salt caused scum-like films to be formed at speeds down to 53 r.p.m. for 0.05 per cent solutions of protein. The thickness of the recovered protein film was approximately the same as that which formed at 94 r.p.m. and 0.06 per cent protein solution without zinc ions. Neurath and Bull (1938) in the review paper dealing with the effect of electrolytes on the surface tension of protein solutions suggested that electrolytes decreased the electrostatic repulsion between protein molecules, thus allowing them to approach more closely on the

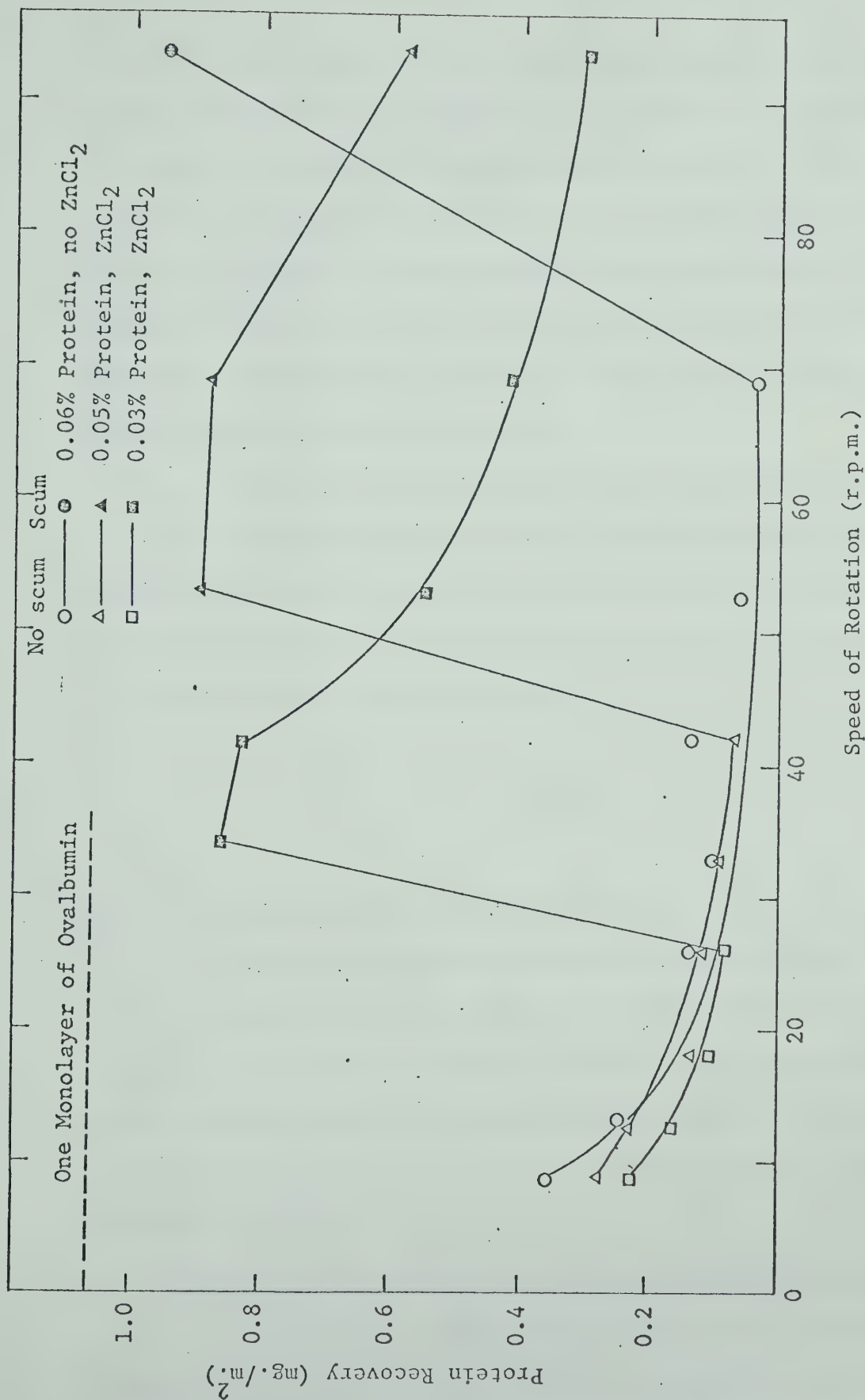


Figure 15.--Effect of zinc chloride and speed of rotation on protein recovery of ovalbumin 1 in buffered solutions (pH 4.8). No corrections have been made for the effect of protein in the solution remaining on the roller when the roller was removed from the tray.

surface of the solution. This almost certainly would have a significant effect on both surface strength and the adhesion of protein film to roller, and hence the presence of cations would be expected to have a marked effect on the speed at which scum formation takes place. For the 0.03 per cent solution, a stable scum-like film was observed at 33 r.p.m., whereas it was not observed at this speed for 0.05 per cent solution. Therefore concentration of protein, as well as the presence of zinc ions, affect the scum formation phenomenon.

The effect of addition of ZnCl_2 (0.05 per cent) to 0.37 and 0.57 per cent solutions of ovalbumin 1 was also studied. Scum was not observed in either solution in the range of speed up to 191.9 r.p.m. It appears then that the lower the concentration of protein, the lower is the speed at which scum is observed.

PART II

1. Differences between Ovalbumin 1 and 2

In several runs during preliminary investigation on the effect of roller speed on recovery of ovalbumin 2 (approximately 0.1 per cent) with device 2, scum was observed at the lowest speed of 18.8 r.p.m. instead of at 94 r.p.m. only as for ovalbumin 1 with device 1. A number of experiments were carried out to determine the reason for the different behaviour.

a. Effect of roller speed on recovery of ovalbumin 2 in device 1

A solution of approximately 0.1 per cent ovalbumin 2 was examined in device 1. The scum point was also observed at 18 r.p.m. This indicated that the change in scum-formation behaviour was not due to differences between the rollers or devices.

b. Effect of roller speed on recovery of ovalbumin 1 in device 2

A solution of approximately 0.1 per cent ovalbumin 1 was used in device 2. The scum point was observed at speeds in the range 54.4 to 76.4 r.p.m. This was similar to ovalbumin 1 in device 1, although the scum point was somewhat lower. This showed that the equipment might possibly have some effects. It was felt, however, that difference between the two batches of protein was more important, and hence all further studies were done using device 2.

c. Effect of roller speed on recovery of ovalbumin 1 and 2 in device 2

Protein recovery (mg./m.^2) is shown in Table 2 as a function of roller speed for 0.06 and 0.32 per cent solutions of ovalbumin 1, and for 0.07 and 0.33 per cent solutions of ovalbumin 2. For ovalbumin 1 (0.06 per cent solution) the scum formation was observed at speeds greater than 66.0 r.p.m., but scum was not observed at any speed with the 0.32 per cent solution. This was similar to the results obtained with device 1 (section 3, page 28). For ovalbumin 2, the scum was observed at every speed both at 0.07 and 0.33 per cent solutions. The protein recovery of ovalbumin 1 when no scum was observed was less than that of ovalbumin 2 at the same speed of rotation. When the scum was observed in ovalbumin 1 system, the protein recovery was approximately the same as obtained from ovalbumin 2 for the same concentration.

d. Effect of roller speed on recovery of globulin fraction

Polyacrylamide gel electrophoretograms of ovalbumin 1 (Figure 16) showed one prominent band and some other fainter bands beside the three bands of ovalbumin A1, A2, and A3, which were the only bands present in the electrophoretograms of ovalbumin 2. The prominent band was assumed to be globulin. The differences in behavior of

Table 2.--Comparison of protein recoveries for ovalbumin 1, 2, 3, and globulin fraction
(all solutions buffered at pH 4.8).

Speed of rotation (r.p.m.)	Period of rotation (sec.)	Protein recovery (mg./m. ²)					
		Ovalbumin 1		Ovalbumin 2		Globulin fraction	
		0.06%	0.32%	0.07%	0.33%	0.05%	0.20%
18.8	60	1.53 ^a	4.87 ^a	1.59	6.02	1.09	3.69
28.9	60	0.78 ^a	2.90 ^a	1.27	5.30	1.06	2.65
42.9	60	0.48 ^a	2.13 ^a	1.24	3.64	0.88	1.83
54.4	60	0.44 ^a	1.68 ^a	1.03	2.81	0.72	1.58
66.0	60	0.37 ^a	1.28 ^a	0.94	2.75	0.70	1.35
76.4	60	1.06	1.10 ^a	0.87	2.45	0.64	1.22
97.9	30	1.30	2.01 ^a	1.03	3.34	0.76	1.58
124.4	30	1.18	1.66 ^a	0.94	2.98	0.65	1.25
150.9	15	1.20	1.83 ^a	1.28	3.26	0.82	2.60
174.5	15	1.11	2.25 ^a	1.18	2.98	0.76	1.78
191.9	15	1.05	2.24 ^a	1.08	3.07	0.72	1.69
							0.07%

^a No scum observed

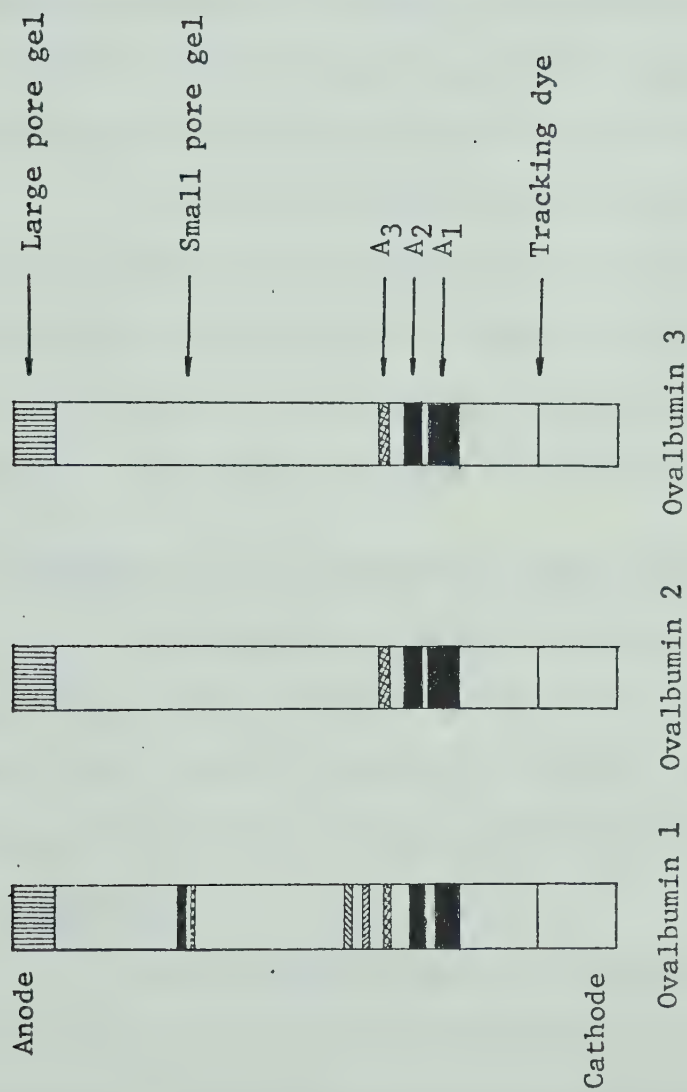


Figure 16.--Polyacrylamide gel electrophoretograms of ovalbumin 1, 2, and 3 in tris-glycine buffer, pH 8.3, stained with 0.05 per cent Coomassie Blue R 250 in 10 per cent trichloroacetic acid.

ovalbumin 1 and 2 were thought to be due to the "globulin impurity". Therefore solutions of globulin in buffer at pH 4.8 of approximately 0.05 and 0.2 per cent were prepared and protein recovery vs. roller speed experiments were carried out. The results are shown in Table 2. No standard curve was available for globulin-concentration measurement, and the curve for ovalbumin 2 was used for approximate determinations. Scum was observed in both solutions at every speed of rotation studied.

If the protein giving the third band was globulin then it would appear that some interaction took place between the ovalbumin and globulin to give properties shown by neither alone. Alternatively, the material giving the third band could have been a protein other than globulin. No attempts were made to identify the impurities.

2. Determination of Thickness of Liquid Layer on Roller Surface

The proposed mechanisms of protein recovery predict that a far thicker layer of liquid is carried over the roller when scum is being formed, than when scum is not being formed. To test this hypothesis, several experiments were performed:

a. Ovalbumin 1

Table 3 shows the amount of liquid film on the upper half of the roller surface, with and without scum formation, for a solution of ovalbumin 1 (approximately 0.1 per cent solution). The amount of liquid film at 18.8 r.p.m. when no scum was observed showed no significant change as the period of rotation was increased. Similarly, speeds of rotation did not appear to have any significant effect on the liquid layer for the series of runs in which scum was formed. The average amount of liquid when scum was observed was higher than that when no

Table 3.--Amount of liquid layer on the upper half of roller surface at several speeds and periods of rotation for ovalbumin 1 (approximately 0.1 per cent) in buffered solution at pH 4.8.

Speed of rotation (r.p.m.)	Liquid (mg) (approx. time when scum observed)			
	15 sec ^a	30 sec ^a	1 min ^a	2 min ^a 4 min ^a
18.8	157, 150, 142 (no scum)	134, 130, 142 (no scum)	143, 130, 130 (no scum)	140, 151, 169 (no scum) 140, 153, 143 (no scum)
36.8				134, 140 (no scum)
54.4				174, 181 (scum, 1 min)
76.4		168, 175 (scum, 6 sec)		
66.0		175, 146, 162 (scum, 10 sec)		
54.4		168, 183, 171 (scum, 8 sec)		
36.8		177, 178, 182 (scum, 15 sec)	171 (scum, 13 sec)	

^a Period of rotation

scum was seen. Application of t-test to the data showed that the difference was significant at the 0.1 per cent level.

The data for speeds greater than 36.8 r.p.m. are consistent with the proposed mechanism for formation of scum. The average thickness of the liquid layer remaining on the roller for the runs in which scum was formed was approximately 0.002 cm., which was equal to that required for a single monolayer (page 33). Some drainage would have occurred during the time the roller was stopped and the time it was weighed, so that the actual liquid layer thickness during rotation was somewhat greater than this minimum value.

The amount of liquid layer on the upper half of the roller surface for a solution of ovalbumin 1 (approximately 0.6 per cent) is shown in Table 4. As the speed of rotation was increased the amount of liquid layer also increased. All runs were of 2-minute periods of rotation. No scum was observed in this series of runs. These results differ from those obtained from 0.1 per cent solution in which the amount of liquid layer was essentially independent of speed. Neurath and Bull (1938) reported in a review paper on the effect of protein concentration on the surface tension of protein solutions that, in general, as the protein concentration is increased the surface tension decreases. This change of surface tension could well cause the difference in the amounts of liquid layer at the higher concentrations.

The dynamic surface tension at the meniscus and on the liquid layer on the roller depends on the amount of protein which has diffused into the surface. The amount of protein which diffuses to the surface depends on the speed of rotation, and hence a distinct effect of speed on the amount of liquid layer is quite reasonable.

Table 4.--Amount of liquid layer on the upper half of roller surface at several speeds of rotation for ovalbumin 1 (approximately 0.6 per cent) in buffered solution at pH 4.8.

Speed of rotation ^a (r.p.m.)	Liquid film ^b (mg.)
18.8	130
36.8	143
54.4	173
76.4	171
97.9	187
124.4	219
150.9	222
191.9	206

^a Period of rotation 2 min. at each speed

^b No scum observed throughout the entire speed range

In the experiments reported in Table 3 it is seen that the runs which were done toward the end of the series showed more rapid scum formation than identical runs performed earlier in the series. The final set of runs at 36.8 r.p.m. showed rapid scum formation, while earlier runs at the same speed showed no scum even after a 4 minute period of rotation. Table 5 shows the times at which scum first appeared for a further series of runs. From this data and that in Table 3, it appears that the phenomenon of scum formation may depend on the history of the solution, i.e., how long the solution has been used with the device and which speeds and intervals of rotation have been applied to the solution.

b. Ovalbumin 2

Table 6 shows the amount of liquid layer remaining on the upper half of the roller surface after being rotated at 18.8 r.p.m. for different intervals of time using a 0.1 per cent solution of ovalbumin2. Two trays were used and three runs were done at each speed. No scum was observed in the runs of 15 second duration at 18.8 r.p.m. The amount of liquid layer was greater than that in all longer runs in which scum was observed. This may be due to an initial transient effect (e.g., initial acceleration of the roller).

Scum was formed in all runs at 150.9 r.p.m. The amount of liquid layer was greater than that at 18.8 r.p.m. There was a slight decrease in the amount of liquid layer as the duration of rotation increased.

A series of runs with six solutions of ovalbumin 2 (concentrations ranging from 0.1 to 2 per cent) were carried out. The speeds of rotation were 18.8 and 150.9 r.p.m. All runs were for 1 minute.

Table 5.--Effect of history of protein solution on scum formation for ovalbumin 1 (approximately 0.1 per cent) in buffered solution at pH 4.8.

Day	Period of rotation (Approx. time when scum observed)				
	18.8 rpm	36.8 rpm	54.4 rpm	66.0 rpm	76.4 rpm
1	15 sec (no scum)	2 min (no scum)	2 min (scum, 1min)		30 sec (scum, 6sec)
				30 sec (scum, 9sec)	
			30 sec (scum, 9sec)		
		30 sec (scum, 20sec)			
		1 min (scum, 18sec)			
		30 sec (scum, 12sec)			
----- Sample left overnight at room temperature -----					
2		1 min (no scum)			
		2 min (no scum)			
			1 min (scum, 12sec)		
		1 min. (scum, 10sec)			
	4 min (no scum)				

Table 6.--Amount of liquid layer on the upper half of roller surface at several speeds and periods of rotation for ovalbumin 2 (approximately 0.1 per cent) in buffered solution at pH 4.8.

Speed of rotation (r.p.m.)	Liquid film (mg.)			
	15 sec ^a	30 sec ^a	1 min ^a	2 min ^a 4 min ^a
18.8	188, 174, 186	137, 138, 156	127, 131, 120	117, 123, 124
(Tray 1)	(no scum)	(scum)	(scum)	(scum)
18.8	162, 164, 172	147, 138, 151	123, 126, 125	141, 136, 138
(Tray 2)	(no scum)	(scum)	(scum)	(scum)
150.9	201, 213	201, 195	191, 194	111, 108, 112
	(scum)	(scum)	(scum)	(scum)

^a Period of rotation

The amount of liquid layer (as shown in Table 7) decreased, both at 18.8 and 150.9 r.p.m., as concentration of the solution increased. Again, the amount of liquid layer at 150.9 r.p.m. was higher than that at 18.8 r.p.m. at the same concentration. Although some distinct trends are apparent in the data, the effects of the type of ovalbumin, concentration of the solution, speed and period of rotation on the amount of liquid layer on the roller surface are not yet clearly understood.

3. Effect of the Depth of Solution on Protein Recovery

It seems probable that the angle of contact at point between the surface of the roller and the surface of the solution might affect the rate of protein recovery. This angle of contact varies with the depth of the solution, i.e., the greater the depth of the solution (from the bottom of the container) the larger is the angle of contact.

The effects of three depths of 0.06 per cent ovalbumin 2 solution (1.27, 1.75 and 2.38 cm.) on protein recovery were studied simultaneously in three trays. The results are shown in Table 8. Analysis of variance ⁽¹⁾ showed that the effect of the depth of solution had no significant effect on the recovery of protein.

4. Effect of Ionic Strength of Buffer Solution on Protein Recovery

To evaluate the effect of ionic strength of the buffer solution on protein recovery at various speeds, three series of runs

(1) An analysis of variance calculation was done on an IBM 360/67 computer using an APL library program (ANOVA)

Table 7.--Amount of liquid layer on the upper half of roller surface at two speeds of rotation and five protein concentrations for ovalbumin 2 in buffered solution at pH 4.8.

Protein concentration (per cent)	Liquid film (mg.)	
	Speed of rotation ^a (r.p.m.)	
	18.8 ^b	150.9 ^c
0.1	140, 135, 155, 145	212, 210, 170, 167, 268
0.5	130, 158, 158, 151, 125	217, 199, 187, 231, 205
0.8	112, 128, 111, 123	130, 158, 127, 187, 102
1.0	116, 97	125, 115, 113, 111
2.0	93, 87, 103	124, 108, 124

^a Period of rotation 1 minute

^b Small amount of scum observed

^c Large amount of scum observed

Table 8.--Effect of the depth of solution on protein recovery of ovalbumin 2
(0.06 per cent) in buffered solution at pH 4.8.

Speed ^a of rotation (r.p.m.).	Protein recovery ^b (mg./m. ²)	
	Depth of solution (cm.)	
	1.27	1.75 2.38
18.8	1.05	0.86 1.15
42.9	0.91	1.00 1.04
54.4	0.90	0.89 0.88
76.4	0.80	0.85 0.80
97.9	0.72	0.72 0.76
124.4	0.66	0.65 0.70
150.9	0.68	0.59 0.62
174.5	0.64	0.64 0.60
191.9	0.57	0.54 0.63

^a Period of rotation 1 minute

^b Scum observed at every speed of rotation

with 0.07 per cent ovalbumin 2 at ionic strength 0.016, 0.067 and 0.141 were performed. The data presented in Table 9 shows only a slight variation of the amount of protein recovered. Analysis of variance showed that the effect of ionic strength of the buffer solution was significant at the 2.5 per cent level. Hence, it is not certain whether the observed effect of the ionic strength is real.

5. Characteristics of Ovalbumin 3

At this stage of the experiment more ovalbumin was required and a third batch (ovalbumin 3) was prepared. Polyacrylamide gel electrophoretograms of this preparation were identical to those of ovalbumin 2 (Figure 16). Protein recovery (mg./m^2) as a function of speed (r.p.m.) at 0.08 per cent solution is shown in Table 2 (page 37). These data show that the amount of protein recovered and the scum formation point obtained from ovalbumin 3 were similar to ovalbumin 2 at approximately the same concentration. Therefore ovalbumin 3 is considered to be identical to ovalbumin 2.

6. Effect of Period of Rotation on Protein Recovery

In experiments to determine the effect of speed of rotation on recovery of ovalbumin 2 and 3 from 0.07 per cent solutions, several different periods of rotation were used. When the results were plotted (protein recovery vs. speed of rotation as shown in Figure 17), breaks in the curves were observed at points corresponding to the changes of duration. This was found to be due to significant amounts of undenatured protein in the solution which remained on the roller when it was removed from the tray at the end of each run. This amount of undenatured

Table 9.--Effect of ionic strength of buffer solution (pH 4.8) on protein recovery of ovalbumin 2 (0.07 per cent).

Speed of rotation (r.p.m.)	Period of rotation (sec)	Protein recovery ^a (mg./m. ²)		
		Ionic strength		
		0.016	0.067	0.141
18.8	60	1.79	1.79	1.55
28.9	60	1.57	1.59	1.66
42.9	60	1.43	1.36	1.49
60.0	60	1.33	1.22	1.23
76.4	60	1.21	1.19	1.10
97.9	30	1.59	1.36	1.24
124.4	30	1.16	1.24	1.10
150.9	15	1.49	1.46	1.40
174.5	15	1.48	1.50	1.33
191.9	15	1.37	1.15	1.26

^a Scum observed at every speed of rotation

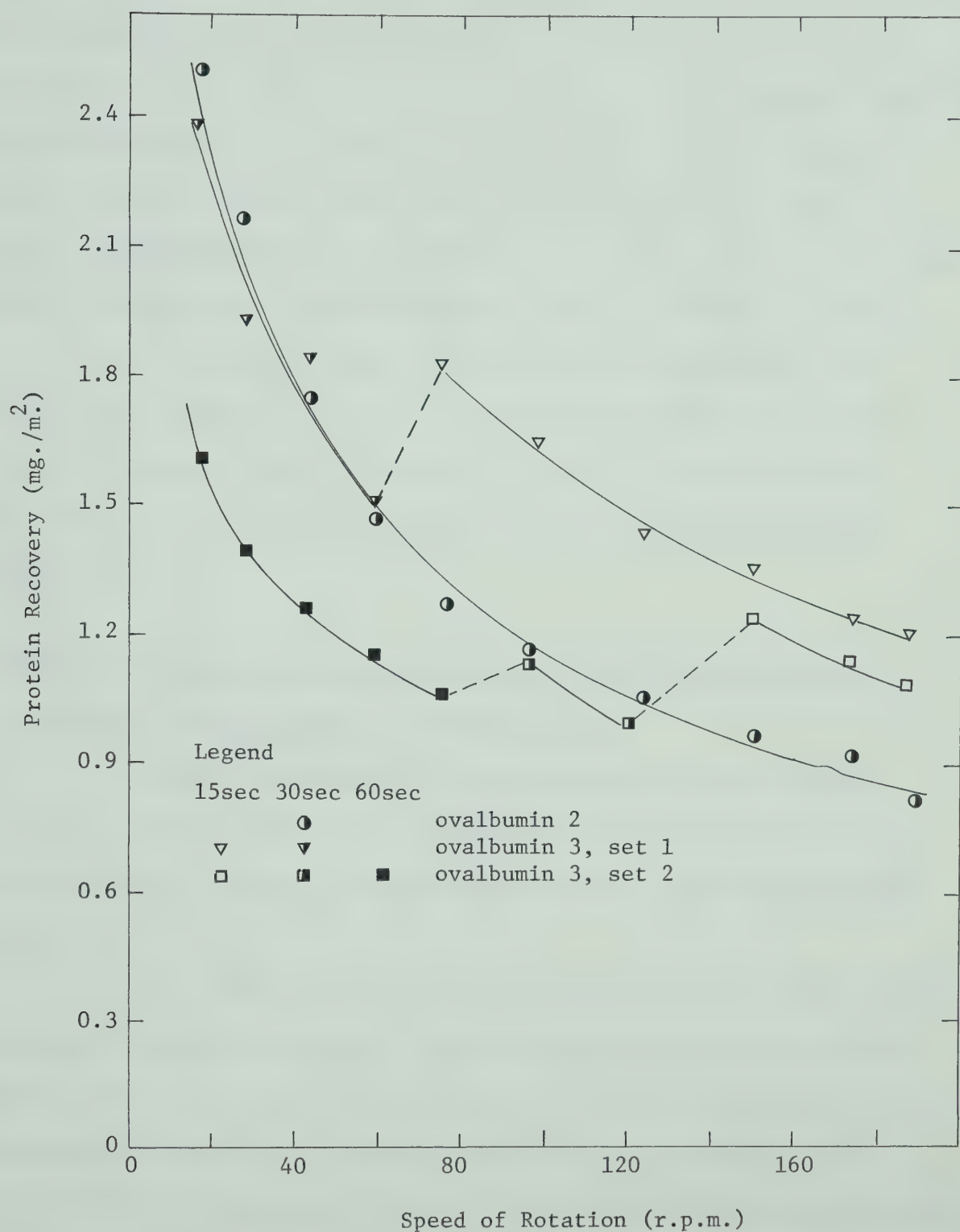


Figure 17.--Effect of period of rotation on protein recovery of ovalbumin 2 and 3 in buffered solutions (pH 4.8). Speed range 18.8-191.9 r.p.m. Concentration 0.07 per cent protein.

protein is approximately the same for all runs at a single concentration because the amount of solution remaining on the roller is more or less independent of the length of the run. The effect of the presence of the undenatured protein is to make the value of protein recovery (mg./m.^2) greater than it should be. With the longer runs which give larger amounts of denatured protein, the effect is much less than it is with the shorter runs which give relatively small amounts of denatured protein.

This observation means that results from experiments which did not take account of the protein in the solution adhering to roller are somewhat greater than they should be. Most of these experiments used uniform periods of rotation, and hence the trends observed will still be valid.

7. Effects of Roller Speed and Concentration of Solution on Recovery of Protein from Solutions of Ovalbumin 1 and 3

Two series of protein recovery experiments were conducted to evaluate the main effects, i.e. speed of rotation and concentration of the solution. The first series used ovalbumin 3 and the second used ovalbumin 1.

In the ovalbumin 3 series eight speeds and three concentrations were used, and in the ovalbumin 1 series, seven speeds and three concentrations were used. Two different periods of rotation were used at each combination of speed and concentration to check the validity of the correction for the adhering solution. Three trays were run simultaneously at each combination of speed of rotation, concentration of solution and period of rotation.

a. Ovalbumin 3 experiments

Table 10 shows the periods of rotation used in the ovalbumin 3 runs. At the lowest speed (9 r.p.m.) and with the most dilute solution (0.075 per cent protein), it was necessary to run for longer periods than at higher speeds and with more concentrated solutions, in order to obtain measurable amounts of denatured protein.

Table 11 shows the protein recovered (mg./m.^2) for the ovalbumin 3 experiment. Scum was observed in all runs. The results of the analysis of variance of the data shown in Table 11 are summarized in Table 12. In the analysis of variance the effect of period of rotation was evaluated on the basis of long and short periods, without regard to the actual magnitude of the period of rotation. Speed, concentration and time (i.e. period of rotation) are all significant at the 0.005 probability level. The probability level for the effect of tray is between 0.025 and 0.050.

All second and third order interactions involving the effect of tray position have probability levels greater than 0.05 and hence are considered to be not significant. All other interactions had probability levels less than 0.005, and hence are considered very significant.

The probability level of the third order interaction speed x concentration x time (less than 0.005) indicates that the effect of time depends on the combination of speed and concentration. To evaluate the effects of time and speed more fully, analyses of variance were done for each of the three concentrations separately. The results of these analyses of variance are shown in Table 13. Again the effect of speed of rotation on protein recovery is very significant (all probability levels less than 0.005). The effect of position of tray is not very

Table 10.--Summary of periods of rotation for ovalbumin 3 runs.

Speed of rotation (r.p.m.)	Period of rotation	Period of rotation (sec)		
		0.075% solution	0.34% solution	0.88% solution
9.0	1	90	90	90
	2	60	60	60
18.8	1	40	30	30
	2	25	15	15
27.5	1	40	30	30
	2	25	15	15
42.9	1	40	30	30
	2	25	15	15
60.0	1	40	30	30
	2	25	15	15
84.0	1	40	30	30
	2	25	15	15
131.3	1	40	30	30
	2	25	15	15
191.9	1	40	30	30
	2	25	15	15

Table 11.--Protein recovery as a function of speed, concentration, period of rotation, and position of tray for ovalbumin 3 in buffered solution at pH 4.8.

Speed of rotation (r.p.m.)	Period of rotation (t)	Recovery of protein (mg./m. ²)								
		0.075% solution			0.34% solution			0.88% solution		
		Tr 1	Tr 2	Tr 3	Tr 1	Tr 2	Tr 3	Tr 1	Tr 2	Tr 3
9.0	1	0.88	1.04	0.78	0.57	0.64	0.76	3.18	2.55	4.78
	2	0.72	0.84	0.56	0.45	0.59	0.57	3.65	4.47	5.30
18.8	1	0.67	0.62	0.61	0.81	0.80	0.86	3.97	3.48	4.35
	2	0.54	0.63	0.53	0.47	0.50	0.52	6.83	6.66	7.59
27.5	1	0.60	0.88	0.70	0.80	0.88	0.64	3.03	3.04	3.00
	2	0.76	0.60	0.68	0.54	0.49	0.59	6.25	5.79	5.98
42.9	1	0.63	0.66	0.66	0.66	0.65	0.75	2.08	2.30	2.35
	2	0.69	0.74	0.93	0.60	1.31	1.08	3.73	4.40	4.21
60.0	1	0.69	0.80	0.74	0.52	0.71	0.75	1.77	1.98	1.88
	2	0.60	0.68	0.67	0.97	1.13	1.12	4.69	4.31	5.01
84.0	1	0.80	0.80	0.72	0.85	0.85	0.83	1.20	1.35	1.40
	2	0.72	0.62	0.65	0.91	1.35	1.22	2.38	2.11	2.07
131.3	1	0.80	0.90	0.73	0.90	0.72	0.89	1.06	1.40	1.11
	2	0.69	0.86	0.82	1.00	0.98	0.96	1.81	2.70	2.21
191.9	1	0.71	0.88	0.88	0.89	0.85	0.87	0.99	1.11	1.06
	2	0.84	0.91	0.86	0.70	0.91	0.91	1.43	1.49	1.57

Table 12.--Summary of results of the analysis of variance of ovalbumin
3 runs for all concentrations of protein.

Source of variation	Probability Level
	0.075, 0.34, 0.88% protein
Speed: Concentration; Time: Tray (no replicates)	
Speed	<0.005
Concentration	<0.005
Tray	.025 --.05
Time	<0.005
Interactions:	
Speed x Concentration	<0.005
Speed x Tray	>.05
Speed x Time	<0.005
Concentration x Time	<0.005
Tray x Time	>0.05
Speed x Concentration x Time	<0.005
Speed x Concentration x Tray	> 0.05
Concentration x Tray x Time	> 0.05
Speed x Tray x Time	> 0.05

Table 13.--Summary of results of the analyses of variance of ovalbumin 3 runs for each concentration of protein.

Source of variation	Probability level of significance		
	Protein concentration (per cent)		
	0.075	0.34	0.88
Speed; Time; Tray (no replicates)			
Speed	<0.005	<0.005	<0.005
Tray	0.025-0.05	>0.05	0.01-0.025
Time	>0.05	>0.05	<0.005
Interactions:			
Speed x Tray	>0.05	>0.05	0.005-0.01
Speed x Time	0.0025-0.05	0.015-0.01	<0.005
Tray x Time	>0.05	>0.05	>0.05

significant, with probability levels of 0.025 to 0.050, greater than 0.050, and 0.010 to 0.025 for the 0.075, 0.34 and 0.88 per cent ovalbumin solutions, respectively. The effect of time was only significant for the runs with 0.88 per cent ovalbumin (probability level less than 0.005). The largest differences in protein recovery between the longer and shorter periods of rotation occur at the lower end of the speed range where the amounts of denatured protein are the least (since the total surface area generated is proportional to the speed of rotation). The runs which give the lowest amounts of denatured protein have the greatest ratios of undenatured to denatured protein, and hence these runs will be affected the most by any errors in determination of the amount of liquid film remaining on the roller after the run.

The speed $\times$ tray interaction for the highest concentration has a probability level between 0.005 and 0.010 (Table 13). The reason for this is not clear. The significant speed $\times$ time interaction (probability level less than 0.005) arises because the greatest effect of time occurs in the lower half of the speed range.

Figure 18 depicts the average rate of protein recovery (mg./min.) as a function of speed of rotation of the roller for each of the three concentrations of ovalbumin 3. The rate of recovery is approximately proportional to speed for both the 0.075 and 0.34 per cent solutions, but the rate of recovery for the 0.88 per cent solution is a nonlinear function of speed with decreasing slope at higher speeds, which indicates that there may be a maximum rate of recovery at speeds higher than those used in this study. These data are quite similar to those of Bull (1937). Bull and Neurath (1937) discussed the decreasing effect of speed on solutions with higher concentration. They suggested that

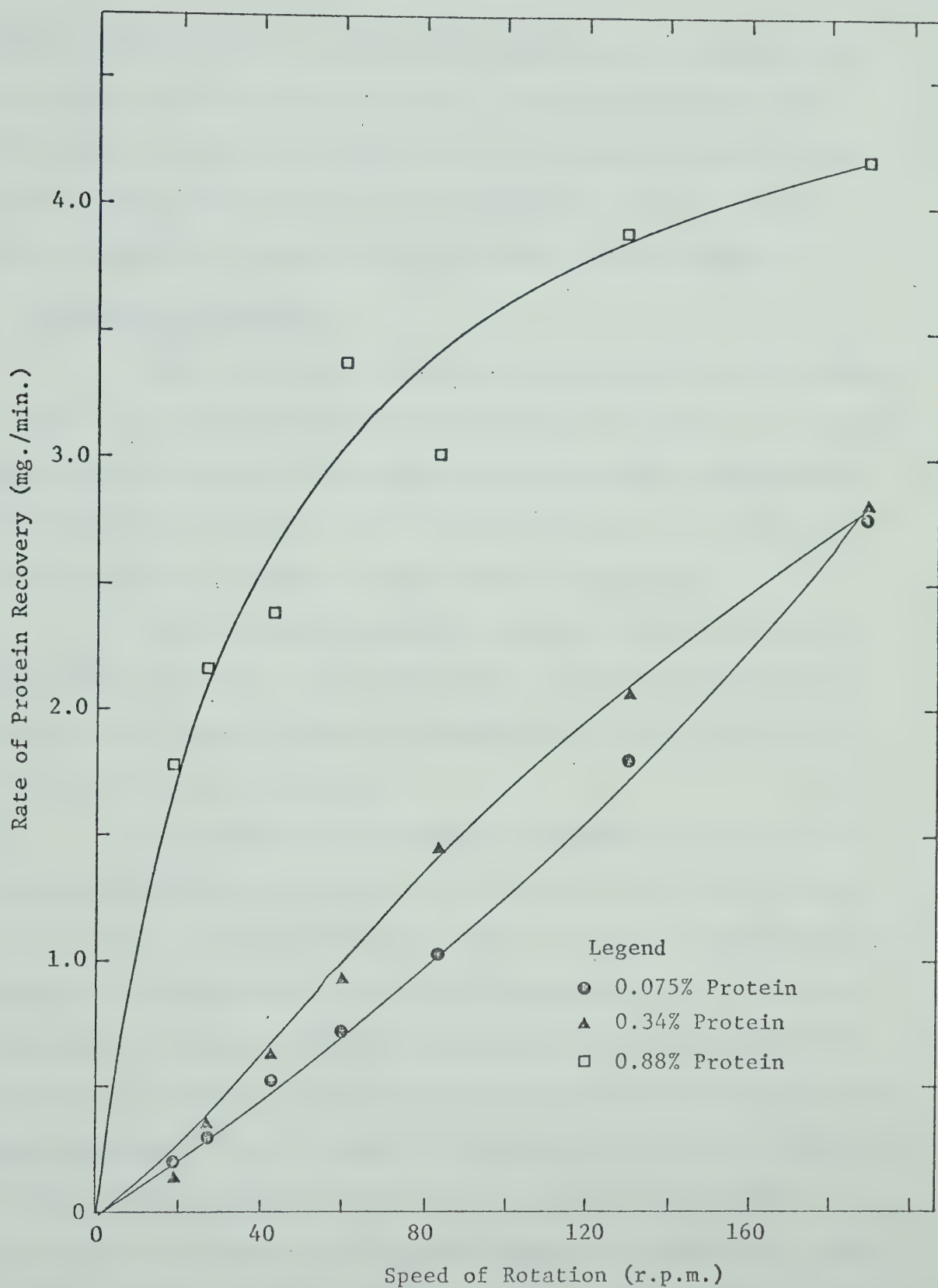


Figure 18.--Effect of speed of rotation on rate of protein recovery of ovalbumin 3 in buffered solution (pH 4.8) for several concentrations of protein. Rate of protein recovery corrected for protein in adhering solution.

the rate of spreading of protein would presumably be inhibited owing to a smaller area in which to spread. At high concentration, the electrostatic attraction between the zwitter ionic groups at the isoelectric point would increase the intramolecular cohesion forces, thus decreasing the ease of spreading rather than increasing it.

b. Ovalbumin 1 experiments

Table 14 shows the periods of rotation used in the ovalbumin 1 runs. In the runs in which scum was formed, the periods of rotation were 30 and 15 seconds for the long and short periods, respectively. These shorter periods were used to avoid decreases in concentration of the solution by removal of large amounts of protein.

Table 15 shows the protein recovered in mg./m.^2 for the ovalbumin 1 experiments. The only runs in which scum formation was observed were those at speeds of rotation of 60 r.p.m. and greater with the 0.05 per cent solution.

The results of the analyses of variance for the two concentrations at which scum formation did not take place (i.e. 0.35 and 0.58 per cent protein) are shown in Table 16. Speed, concentration, and time were all significant at the 0.005 probability level. The very significant effects of speed and concentration were as expected but the effect of time (i.e. period of rotation) was much more significant than anticipated. At all speeds the recoveries of protein in the runs of long period (period 1 in Table 15) are less than the protein recoveries in the runs of short period (period 2 in Table 15.). Much longer periods of rotation were used in the runs which did not give scum than were used in the runs in which scum formed, in order to obtain larger amounts of denatured protein. In spite of this, the amount of

Table 14.--Summary of periods of rotation for ovalbumin 1 runs.

Speed of rotation (r.p.m.)	Period of rotation	Period of rotation (min.)		
		0.05% solution	0.37% solution	0.58% solution
18.8	1	10	10	10
	2	5	5	5
27.5	1	10	10	10
	2	5	5	5
42.9	1	10	10	10
	2	5	5	5
60.0	1	0.5 ^a	10	10
	2	0.25 ^a	5	5
84.0	1	0.5 ^a	10	10
	2	0.25 ^a	5	5
131.3	1	0.5 ^a	10	10
	2	0.25 ^a	5	5
191.9	1	0.5 ^a	10	10
	2	0.25 ^a	5	5

^a Scum formed

Table 15.--Protein recovery as a function of speed, concentration, period of rotation, and position of tray for ovalbumin in buffered solution at pH 4.8.

Speed of rotation (r.p.m.)	Period of rotation	Recovery of protein (mg./m. ²)								
		0.05% solution			0.37% solution			0.58% solution		
		Tr 1	Tr 2	Tr 3	Tr 1	Tr 2	Tr 3	Tr 1	Tr 2	Tr 3
18.8	1	0.043	0.037	0.039	0.117	0.086	0.118	0.179	0.182	0.175
	2	0.104	0.090	0.096	0.170	0.173	0.181	0.288	0.241	0.238
27.5	1	0.027	0.024	0.026	0.051	0.055	0.050	0.103	0.114	0.106
	2	0.064	0.075	0.068	0.118	0.118	0.120	0.181	0.191	0.181
42.9	1	0.026	0.027	0.022	0.045	0.041	0.040	0.064	0.079	0.067
	2	0.045	0.055	0.039	0.125	0.075	0.086	0.104	0.116	0.120
60.0	1	0.79 ^a	0.71 ^a	0.83 ^a	0.033	0.033	0.034	0.050	0.042	0.056
	2	0.92 ^a	1.05 ^a	1.05 ^a	0.058	0.053	0.060	0.091	0.081	0.087
84.0	1	0.83 ^a	0.87 ^a	0.90 ^a	0.032	0.029	0.030	0.034	0.036	0.041
	2	0.81 ^a	0.82 ^a	0.98 ^a	0.059	0.060	0.056	0.073	0.075	0.073
131.3	1	0.79 ^a	0.80 ^a	0.81 ^a	0.022	0.020	0.020	0.027	0.023	0.027
	2	0.76 ^a	0.70 ^a	0.79 ^a	0.040	0.040	0.043	0.049	0.054	0.054
191.9	1	0.61 ^a	0.64 ^a	0.67 ^a	0.020	0.021	0.020	0.019	0.019	0.020
	2	0.67 ^a	0.61 ^a	0.67 ^a	0.033	0.040	0.033	0.041	0.040	0.046

^a Scum observed

Table 16.--Summary of results of the analysis of variance of ovalbumin 1 runs for all concentrations of protein.

Source of variation	Probability of significance
	0.35, 0.58% protein
Speed; Concentration; Time; Tray (no replicates)	
Speed	<0.005
Concentration	<0.005
Tray	>0.05
Time	<0.005
Interactions:	
Speed x Concentration	<0.005
Speed x Tray	>0.05
Speed x Time	<0.005
Concentration x Tray	>0.05
Concentration x Time	>0.05
Tray x Time	>0.05
Speed x Concentration x Tray	>0.05
Speed x Concentration x Time	>0.05
Concentration x Tray x Time	>0.05
Speed x Tray x Time	>0.05

denatured protein is very sensitive to errors in the estimate of the undenatured protein. Examination of the raw data does not indicate clearly whether the significant effect of time arises from errors in the estimate of the amount of undenatured protein or whether it arises from some other effect.

The effect of tray was not significant at the 0.05 probability level.

The only interactions which showed probability levels less than 0.05 were speed x concentration and speed x time (probability levels both less than 0.005). The interaction of speed and concentration is in agreement with the non-scum data on ovalbumin 1 presented in Figure 12, where the effect of speed depends very much on concentration. Examination of the raw data shows that the ratio of undenatured to denatured protein decreases as the speed increases. Hence the significant interaction between time and speed could arise from errors in the estimate of undenatured protein in the solution remaining on the roller after removal from the tray.

Two analyses of variance were done for the 0.05 per cent ovalbumin 1; the first for the four speeds which gave scum formation, and the second for the three speeds which did not give scum formation. The results of these analyses are summarized in Table 17 along with the results from the analysis of variance of each of the two concentrations which did not give scum formation at any speed.

The first column of Table 17 shows the probability levels for the runs which gave scum formation. The effect of speed is very significant (probability level less than 0.005). This is as observed

Table 17.--Summary of results of the analyses of variance of ovalbumin 1 at 0.05 per cent protein, with and without scum formation, and at 0.35 and 0.58 per cent protein, without scum formation.

Source of variation	Probability level of significance		
	Protein concentration (per cent)		
	0.05 ^a	0.05 ^b	0.35 ^c 0.58 ^c
Speed; Time; Tray (no replicates)			
Speed	<0.005	<0.005	<0.005
Tray	0.01-0.025	>0.05	>0.05
Time	0.01-0.025	<0.005	<0.005
Interactions:			
Speed x Tray	>0.05	>0.05	>0.05
Speed x Time	<0.005	<0.005	<0.005
Tray x Time	>0.05	>0.05	>0.05

^a Speeds 60-191.9 r.p.m.; scum formed

^b Speeds 18.8-42.9 r.p.m.; no scum formed

^c All speeds; no scum formed

in the ovalbumin 3 experiments where scum was formed in all runs. The effects of tray and time were significant (probability levels both between 0.010 and 0.025). The reason for the effect of tray is not clear. The correction for undenatured protein is less than the correction required for the runs in which no scum was formed, and hence the effect of time is not as significant as the effect of time in the runs with 0.37 and 0.58 per cent solutions. The very significant speed x time interaction (probability level less than 0.005) arises because the greatest effect of time occurs at the lowest speed. This is very similar to the effect of speed on time observed with the ovalbumin 3 experiments.

The second column of Table 17 summarizes the results of the analysis of variance for the three speeds with 0.05 per cent ovalbumin 1 which did not show scum formation. The probability levels of all main effects and interactions fall in the same ranges as those for the two higher concentrations which did not form scum at any speed. The results of the analyses of variance for these two speeds are shown in columns 3 and 4 of Table 17.

Figure 19 shows the effect of speed of rotation on the average rate of protein recovery (mg./min.). This figure corresponds to Figure 11 (page 27) which shows data uncorrected for adhering solutions. The data of Figure 19 for the runs not giving scum are approximately half to one third the corresponding data of Figure 11, but, the general trends are the same. The rates of recovery of protein with scum formation are quite comparable. This is because the error introduced by the adhering solution is relatively unimportant for the long run used for the data of Figure 11. Comparison of the lowest speeds at which scum forms shows

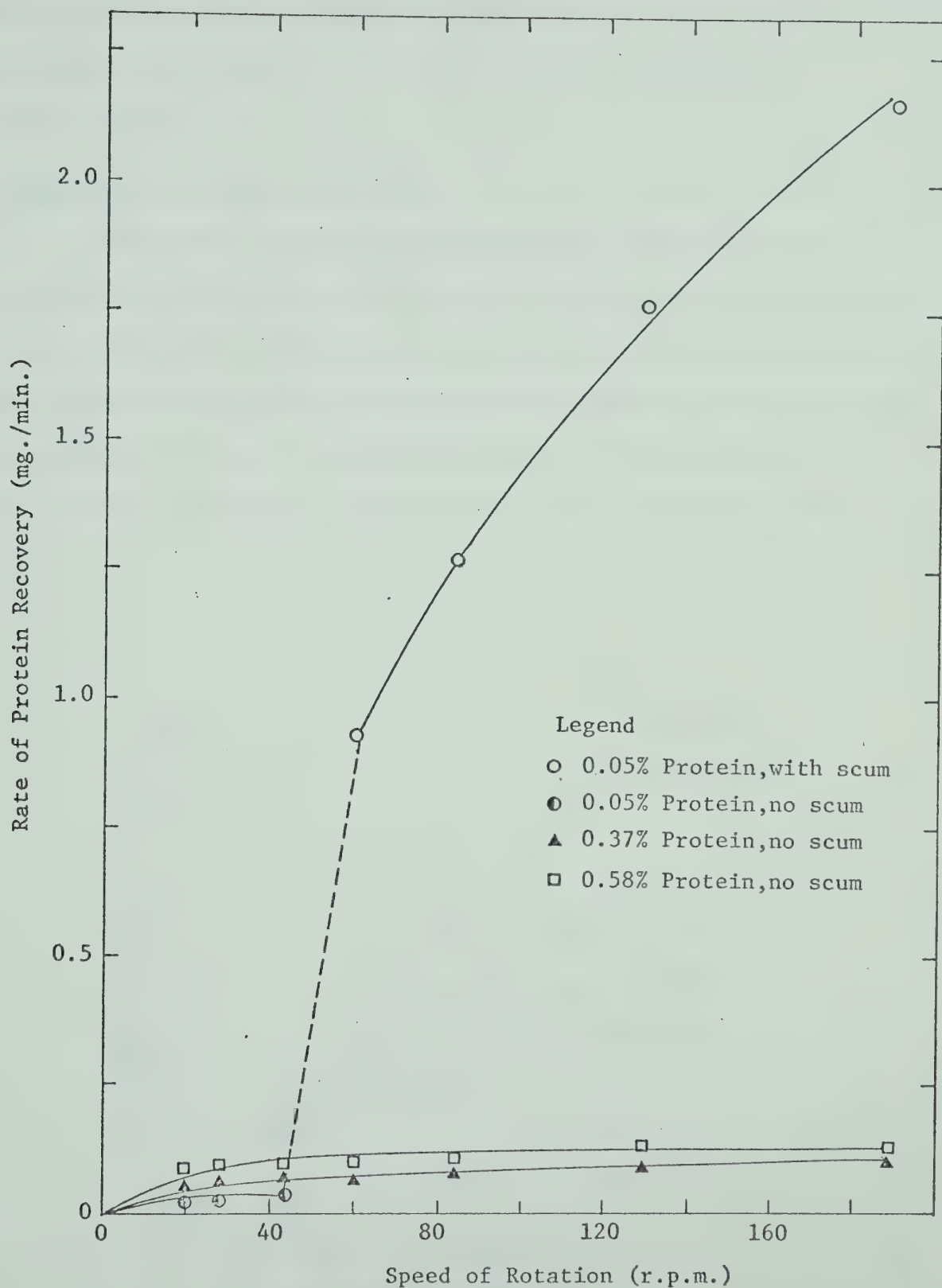


Figure 19.--Effect of speed of rotation on rate of protein recovery of ovalbumin 1 in buffered solutions (pH 4.8) for several concentrations of protein. Rate of protein recovery corrected for protein in adhering solution.

that the concentration of protein has some effect in the absence of zinc chloride as it does in the presence of zinc chloride (see Figure 15, page 34).

c. Comparison with the work of Bull

The present investigation, in general, does agree with the work of Bull (1937) in that the amount of surface denatured protein per unit surface area depends mainly on speed of rotation and concentration. However, none of Bull's data compares with the data of the non scum runs with ovalbumin 1. This is seen quite clearly in a comparison of Figures 20 and 19 which show the rates of protein recovery (mg./min.)

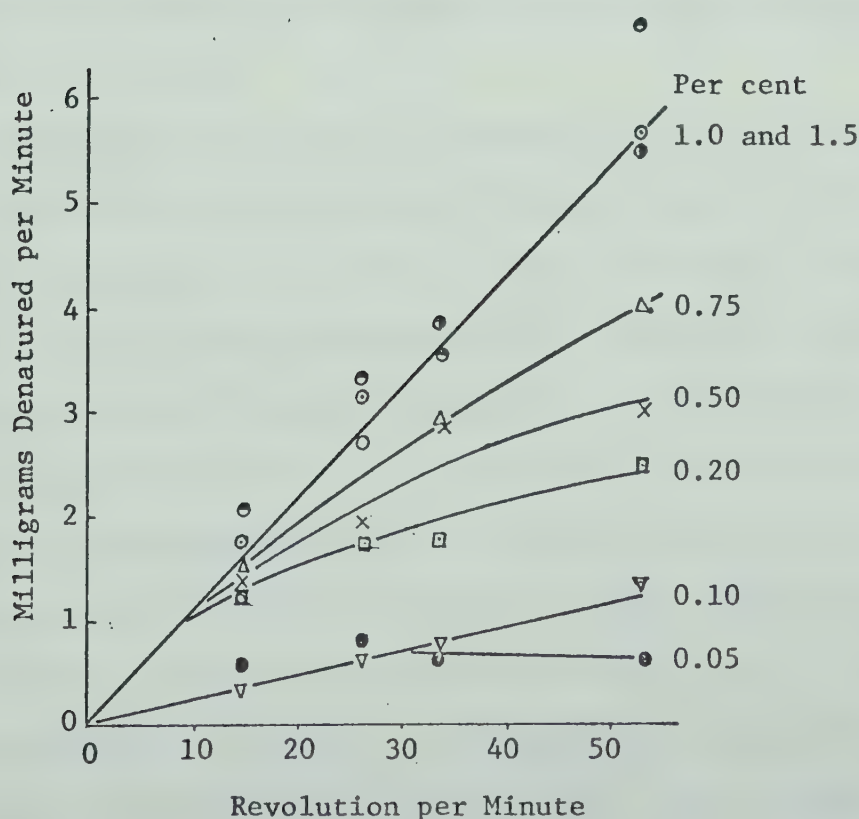


Figure 26.--Amount of denaturation per minute as a function of speed of rotation. From Bull (1937).

as functions of speed of rotation for the various concentrations used in Bull's work, and the corresponding curves for ovalbumin 1, respectively. It should be noted that the surface area per revolution of the rollers used in the two studies are almost identical (this work - 173 cm.^2 ; Bull's work - 171.6 cm.^2). The curve in Figure 19 for the runs which gave scum formation (open circles) can be extrapolated to the origin. Bull's data for 0.05 and 1.10 per cent protein fall in this low speed range, and his curve for these data is quite close to the extrapolated line of Figure 19. His data for higher concentrations give rates of protein recovery ranging up to 6 mg./min. at 50 r.p.m. whereas the rates of protein recovery for the higher concentrations of ovalbumin 1 are at most 0.25 mg./min.

Figures 21 and 22 show the average protein recoveries (mg./m.^2) for various concentrations of ovalbumin 1 and 3, respectively, as functions of protein concentration. These curves can be compared with Bull's curve of amount of denatured per rotation as a function of protein concentration (Figure 4, page 11). The plateau in Figure 4 is approximately 5 mg./m.^2 . The points in Figure 21 for the runs which gave scum formation are of the same order as the lower end of Bull's curves, but the data in Figure 21 for the runs showing no scum formation do not correspond with any data in Bull's figure. The points at low concentrations in Figure 22 are somewhat lower than those at the lower concentrations in Figure 4, but the effect of an increase in concentration on the protein recovery is quite similar to that reported

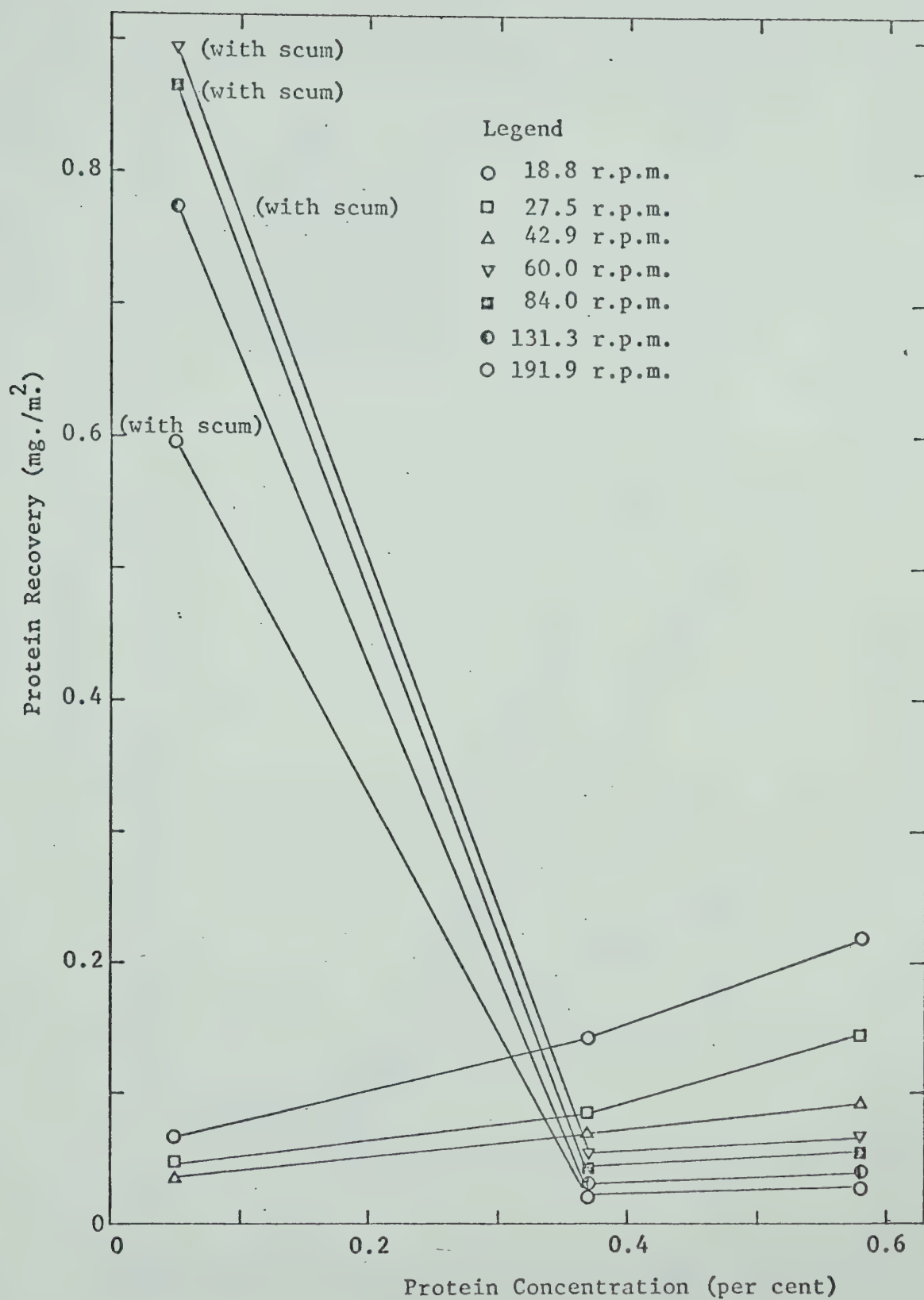


Figure 21.--Effect of protein concentration on protein recovery of ovalbumin 1 in buffered solutions (pH 4.8) at several speeds of rotation. Protein recovery corrected for protein in adhering solution.

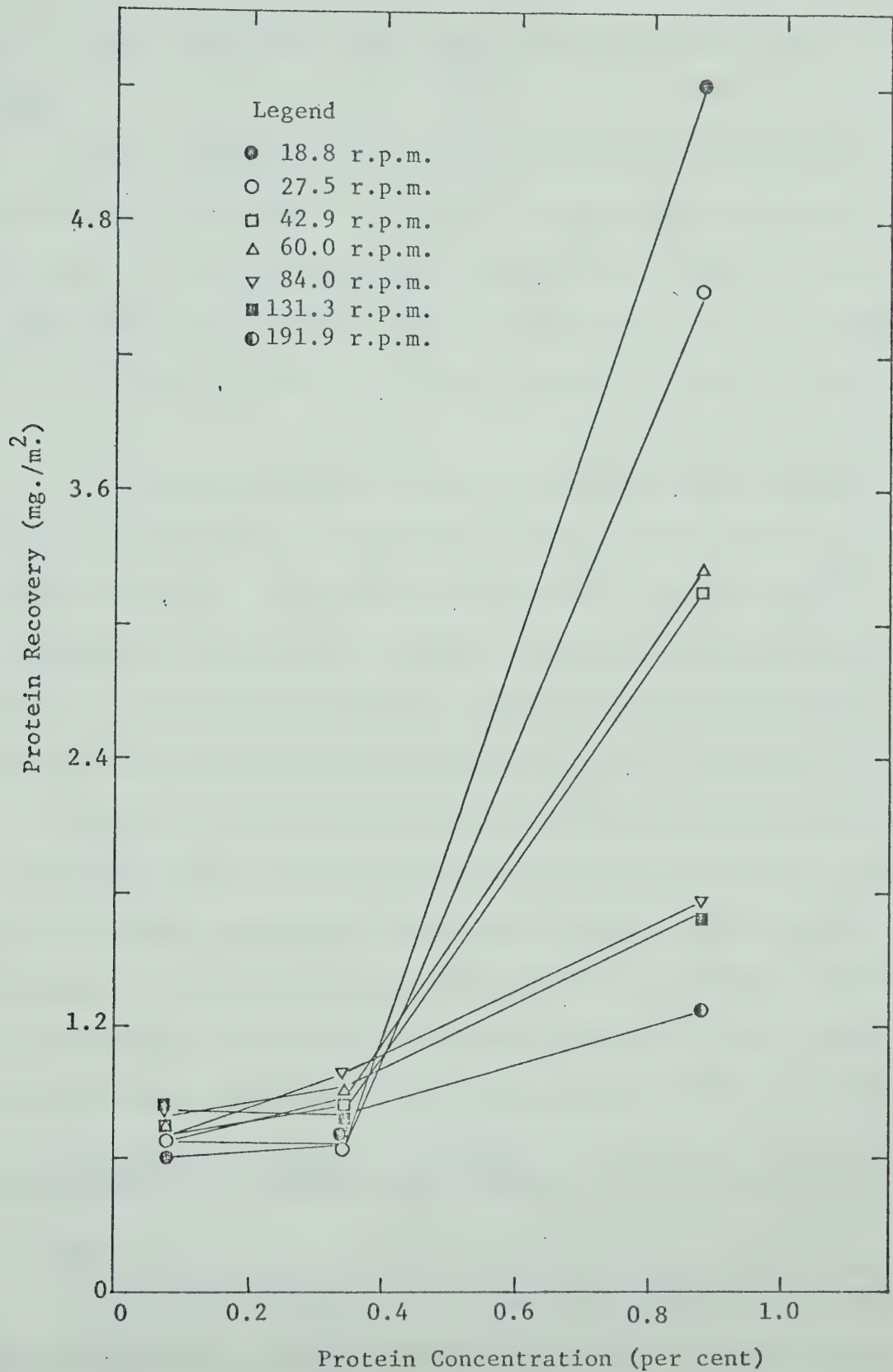


Figure 22.--Effect of protein concentration on protein recovery of ovalbumin 3 in buffered solutions (pH 4.8) at several speeds of rotation. Protein recovery corrected for protein in adhering solution.

by Bull. In both studies the lower speeds gave the higher protein recoveries.

Bull's curves show sharp decreases in slope at about 0.2 per cent protein concentration. The three concentrations used in the present study do not give enough data to define this change of slope. It is clear that if this abrupt change of slope occurs with the systems used in this study, it occurs at a higher concentration than in Bull's work.

Bull does not indicate in his paper whether scum formation was observed. The comparison between his work and the present work would indicate that his system was operating under conditions which gave scum formation although the apparatus was such that the denatured protein was wiped from the roller with cotton material and hence no visible scum could accumulate on the downstream side of the roller.

Myers and Harkins (1937) have shown that different materials have considerable different effects on the properties of adhering surface films. The different materials used in Bull's study and the present work may therefore account for the absence of no-scum operation in Bull's study. Alternatively the absence of no-scum operation in Bull's study may have been due to differences in the proteins used in the two studies.

8. Applications of the Rotating Roller Device to Other Types of Proteins

a. Whey

Fresh whey at pH 4.6, 200 ml. was used to observe the scum formation phenomenon at different speeds of rotation. Although a scum-like film was not observed as in the case of ovalbumin, there appeared

a very small amount of denatured protein on the surface of the solution at the speed of 191.9 r.p.m. after a 10-minute period of rotation. A similar phenomenon was observed when whey adjusted to the isoelectric point of β -lactoglobulin (pH 5.2) was studied.

The effect of the addition 0.1 per cent ZnCl_2 to whey at pH 5.2 was studied. At the speed of 18.8 r.p.m., a small amount of denatured protein appeared on the surface of the solution, but a scum-like film was not observed. At higher speeds, a small amount of scum-like film was observed. The scum did not stay on the surface of the solution, but climbed up on the edge of the tray on the down stroke of the roller. The scum did not deposit onto the roller surface on the removal of the roller from the solution. Because of difficulties in determination of amounts of protein recovered, no quantitative results can be presented. These preliminary studies do show, however, that whey proteins are not as susceptible to surface denaturation as ovalbumin.

b. Serum protein

A similar experiment was also performed with serum protein. At high roller speeds, a scum-like film similar to that of ovalbumin was observed. This scum dissolved almost immediately into the bulk of the solution. At the medium speed of 60 r.p.m., scum was observed clearly. Here, only a portion of the observed scum dissolved, and the rest of the scum was deposited on the surface of the roller when the roller was removed from the solution. This observation showed that the denaturation of serum protein was, to a certain extent, a reversible process. This indicates that its native form is, under proper conditions, a highly stable one. However, it appeared to be possible to recover protein from serum using the technique of rotating roller.

The applications of the rotating roller technique to recovery of protein from whey and serum showed some potential, and suggests that a thorough study of these two types of protein should be made in the future.

CONCLUSIONS AND RECOMMENDATIONS

1. Protein from surface film can be recovered using a rotating roller device, especially ovalbumin which is very susceptible to surface denaturation. This confirms the work of Bull (1937).

2. Rate of protein recovery is higher at the isoelectric point of ovalbumin (pH 4.8) than at pH 6.75. This is as expected from the work of Wu and Ling (1927), and Bull and Neurath (1937).

3. In the system of impure ovalbumin at very dilute solutions, two mechanisms appear to operate in the protein recovery process:

a) At low speeds, the protein layer adheres firmly onto the roller surface. It appears that the roller removes all of the protein from the surface of the bulk liquid and that the rate of recovery of protein is controlled by the rate of diffusion of protein to the free liquid surface.

b) At high speeds, the protein layer does not adhere to the roller surface, but it is ejected to form a stable scum-like film on the surface of the solution on the down side of the roller. It appears that most of the denaturation takes place on a thin film of solution which is carried over on the roller. The rate of recovery with scum formation is much greater than that with no scum formation. Scum formed at all speeds for pure ovalbumin, which shows that the nature of the protein has a very significant effect on recovery using the rotating roller technique.

The presence of solution on the upper half of the roller surface in amounts sufficient to account for the observed quantities of denatured protein has been demonstrated.

4. The characteristics of protein recovery are as follows:

a) The scum-formation point, (i.e. the lowest speed at which scum is formed) depends on the type and concentration of protein, the presence or absence of zinc ions, and to a lesser extent, on the history of the solution, and the mechanical characteristics of the device. Additional (unidentified) fractions in the ovalbumin, high concentration of protein, and the absence of zinc ions all tend to give higher scum-formation point .

b) In the recovery of protein with no scum formation, the rate of recovery is substantially independent of speed, and is approximately proportional to the concentration of ovalbumin. The rates of recovery are much lower than those reported by Bull (1937). This process of recovery of protein without scum formation on the down stream side of the roller was observed for the first time in the present study.

c) In the recovery of protein with scum formation; the rate of recovery depends mainly on speed and concentration. At lower concentrations the amount of ovalbumin recovered (in mg./m^2) is, more or less, independent of speed and is approximately equivalent to a single monolayer of protein. At higher concentrations and low speeds, the amount of protein recovered is equivalent to several monolayers but the amount recovered decreases to approximately one monolayer as the speed is increased.

These general trends observed are quite comparable to those reported by Bull (1937), and it therefore appears that the experiments in Bull's study were done under conditions that give rise to scum formation, even though his equipment prevented the accumulation of the visible scum-like film on surface of the liquid on the downstream side of the roller.

The depth of the immersion of the roller into the solution does not have a significant effect on the rate of recovery in a solution of 0.06 per cent ovalbumin.

The effect of ionic strength of the buffer solution does not have a major effect on the rate of recovery.

d) The effects of period of rotation and position of tray indicate that some obscure factors are involved in either the runs or the determination of the protein recovered as the position of tray (or order of analysis) and length of rotation have a very significant though not a major effect on the protein recovery in some of the runs.

5. A preliminary investigation of recovery of protein from whey (from cow's milk) shows that untreated whey protein is not as susceptible to surface denaturation as ovalbumin. Recovery of protein from beef blood serum appears to be more promising, although the surface denaturation of the protein seems to be a reversible process under the conditions of the preliminary experiments.

On the basis of the results obtained in this investigation the following recommendations for future work can be made.

1. Additional data should be obtained using the roller technique. The effects of higher speeds, addition of zinc ions, and other divalent and trivalent cations at various concentrations, ionic strength and type of buffer system should be evaluated. The method of determination of the amount of recovered protein should be checked by measurements of the change in concentration of the bulk of the solution.
2. Improved techniques should be developed for determination of liquid film carried over the roller surface both with scum and without scum formation.
3. The effects of various physical properties such as surface viscosity, surface tension, characteristics of adsorption of the film on to the roller, and temperature should be studied.
4. Different geometries, such as different sizes of roller and container, and series of discs, etc., should be studied. This would help in optimum system design.
5. When the characteristics of the operation have been evaluated, the recovery of other types of protein such as whey and serum protein should be investigated. This should include a study of pre-treatments of the raw protein solutions, or addition of other surface-active materials, which may improve the recovery of the proteins.

The absolute amounts of protein recovered per unit area are very small. In spite of this, it is the opinion of the present workers that this process can be applied in some forms to the recovery of protein from dilute solutions on an industrial scale. Future studies should be directed toward a better understanding of the scum formation mechanism and the factors which cause scum formation, rather than the mechanism of recovery of protein without scum formation, as the rates of recovery of protein with scum formation are much greater than those without scum formation.

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APPENDIX

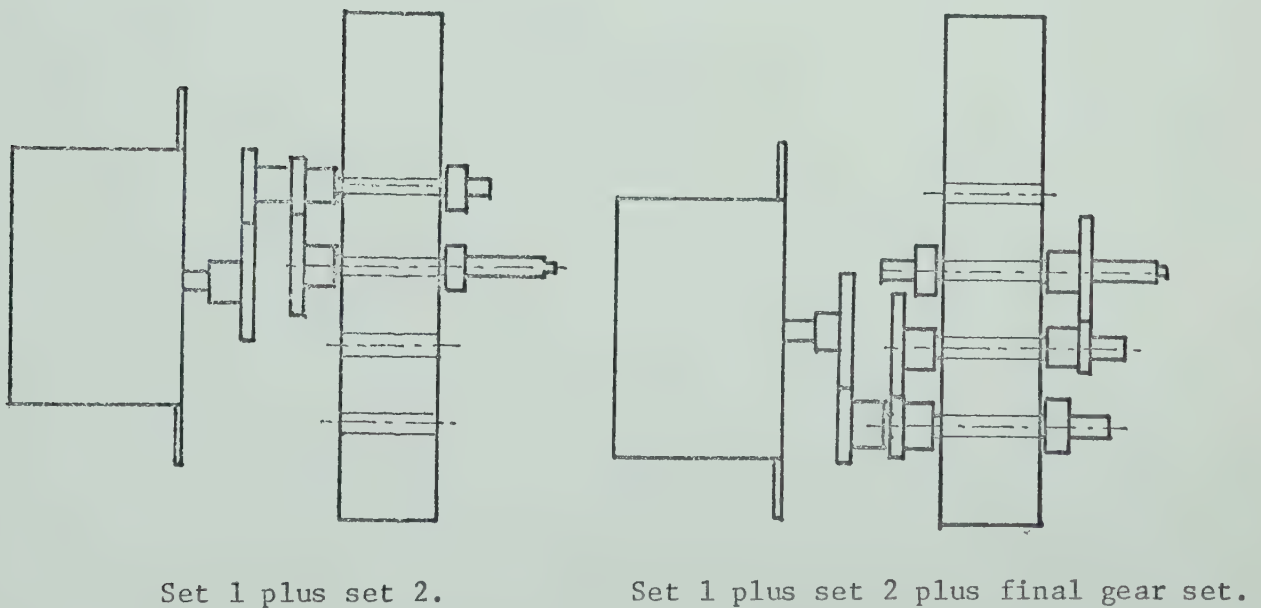
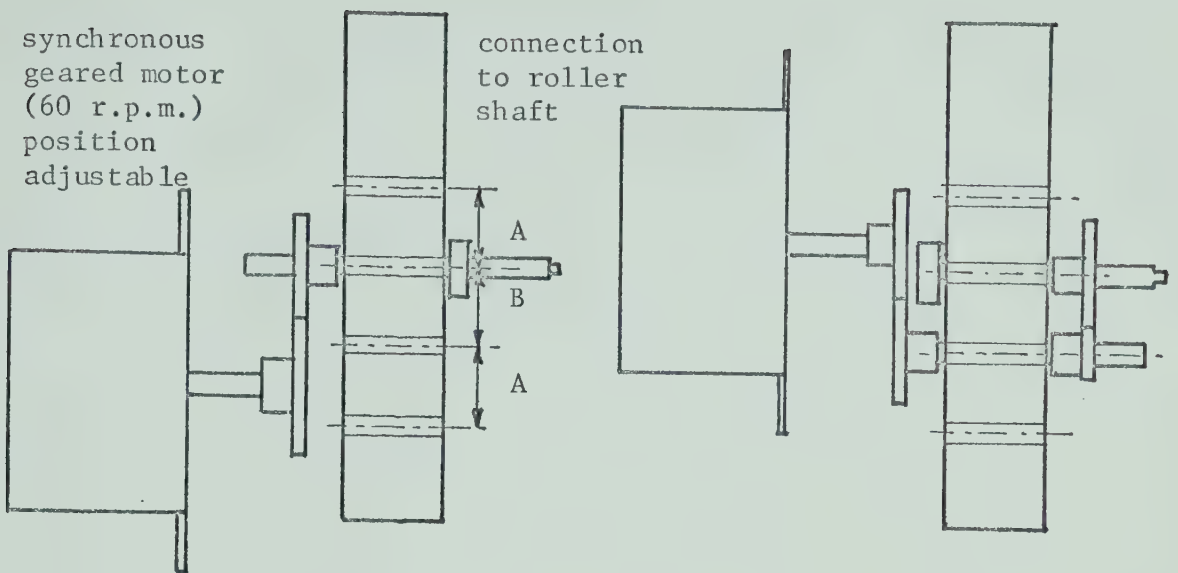


Figure 23 -- Arrangement of gear trains for multi-tray rotating roller device. For gears of 64 pitch the distances between shaft centre lines are $A = 0.750$ in. and $B = 0.719$ in.



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